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THE UNIVERSITY OF ALBERTA

ENDOCRINE RESPONSE OF MALE GOLDFISH TO A FEMALE SEX PHEROMONE, 17α,20B-DIHYDROXY-4-PREGNEN-3-ONE: CHARACTERIZATION AND CENTRAL REGULATION

by

Joseph G. Dulka

C

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1989

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Figures 1-3: Day-night sensitivity of male goldfish to water-borne 17,20B-P. All figures redrawn.

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Figure 5: Influence of a one hour exposure to various doses of water-borne 17,20B-P on GtH secretion in male goldfish.

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ABSTRACT

This study examined the time-course and central regulation of male endocrine responses to the steroidal sex pheromone, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), in goldfish. Approximately 10 h prior to ovulation, females synthesize increased amounts of 17,20B-P to induce final oocyte maturation and then release 17,20B-P to the water as a sex pheromone. Males exposed to 17,20B-P-releasing females or 17,20B-P added directly to aquarium water (10^{-10} M) increase serum gonadotropin (GtH) within 15 min and milt production within 4-6 h. 17,20B-P-induced increases in milt production are dependent on elevated GtH levels since hypophysectomy blocks the milt response to the pheromone. Endogenous testicular 17,20B-P presumably mediates the action of GtH on milt production. The neuroendocrine trigger for 17,20B-P-induced GtH release involves a rapid reduction in dopamine inhibition rather than a stimulation by gonadotropin-releasing hormone. Both the temporal pattern of 17,20B-P release by females and the latency of the GtH and milt responses in males suggest that 17,20B-P is a pheromone that synchronizes milt production with ovulation in goldfish.

The threshold water concentration (10^{-11} M) of 17,20B-P required to induce GtH release in males is similar to that required to evoke electrical responses from the olfactory epithelium. GtH and milt responses to 17,20B-P are dependent on the medial olfactory tracts which project to brain regions that control GtH release.

Male GtH responses to water-borne 17,20B-P were compared to those induced by exposure of males to ovulated or prostaglandin (PG) injected females which release a PG pheromone that stimulates male sexual behavior. Males show circadian differences in endocrine responsiveness to PG-releasing females, such that GtH responses are maximal during scotophase and often absent during photophase, whereas males respond to 17,20B-P at all times of the day. Water-borne 17,20B-P alone elevates GtH release in a reflexive manner while males require behavioral interactions with PG-releasing females before GtH responses can be triggered. The results suggest that the 17,20B-P and PG pheromones operate through different neuroendocrine mechanisms to regulate GtH release in male goldfish. Neither pheromone affects growth hormone release in males. Based on the above results, a model for the study of sex pheromone function in teleost is proposed.

ACKNOWLEDGEMENTS

This thesis is a direct reflection of the training, supervision and assistance I received from my supervisor, Norman E. Stacey. Norm deserves my deepest thanks for being an excellent professor and for providing unlimited support, encouragement and enthusiasm for all my endeavors. The years I have spent with Norm, both in and outside the laboratory, will always be considered the best of times. I am also greatly indebted to Dick Peter who assisted in my training, provided outstanding research facilities and maintained a continuous supply of students and post-doctoral fellows who contributed greatly to the development of my scientific career. I also wish to acknowledge the members of my supervisory committee, Andy Spencer and Steve Harvey, for their time, interest and helpful suggestions regarding my research project. My external examiner, John Wingfield, and examination chairman, Jan Murie, made the defense of this thesis a truly memorable experience.

Perhaps the most enjoyable aspect of my research has been the people that I have been associated with over the past six years. Special thanks goe to Peter Sorensen, Tracy Marchant, Glen Van Der Kraak, Carol Nahorniak and Vance Trudeau for being colleagues, advisors, amateur psychiatrists and extremely good friends. I hope they all realize how much I relied on them throughout the entire ordeal and apologize for the times that I made their lives absolutely miserable! Countless others also deserve my thanks, especially Duff Sloley, Ned Pankhurst, Olivier Kah, Gustavo Somoza, John Chang, Mirka Sokolowska, Yuang-Ping Yuan, Kei-li Yu, Makito Kobayashi, Kuni Suzuki, Hamid Habibi, Teresa Krukoff, Warren Gallin and Jeff Goldberg. My friends in Devon, Jay, Jackie, Pat and Terry deserve special thanks for providing sanctuary, friendship and trust. All these people will be dearly missed but never forgotten.

I would also like to use this opportunity to thank the teachers and professors who inspired me to pursue a career in science. These exceptional people include: Mrs. Brugerman at St. Mary's, Mr. Norman Korwitz at Mentor High School, Cindy Groat at

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11. APPENDIX II

Stacey, N.E., P.W. Sorensen, G.J. Van Der Kraak, and J.G. Dulka. 1989. Direct evidence that 17α,20B-dihydroxy-4-pregnen-3-one functions as a goldfish primer sex pheromone: preovulatory release is closely associated with male endocrine responses. Gen. Comp. Endocrinol. (in press).....217.

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1. GENERAL INTRODUCTION

Chemical communication through the use of pheromones plays an important role in a variety of reproductive functions in fish, although fish also use pheromones for a variety of non-reproductive functions including individual recognition, parent-young interactions, alarm or fright reactions and migration (see reviews by Colombo et al., 1982; Lambert et al., 1986; Liley, 1982; Stacey, 1983; Stacey et al., 1986, 1987). Only reproductive aspects of chemical communication will be considered here. Non-teleost sex pheromone systems also will be considered to illustrate how sex pheromones operate in both the aquatic and terrestrial environments. The range of soluble biochemical compounds which may function as sex pheromones in the aquatic environment far exceed those which must be volatile enough to function as sex pheromones in the terrestrial environment (Wilson, 1975; Stacey et al., 1986; Resink, 1988).

Karlson and Luscher, who introduced the term *pheromone* in 1959, defined them as "substances that are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process". Wilson and Bossert (1963) later modified this definition by classifying different pheromones as either "releasers" or "primers". Sex pheromones are now categorized as releasers if they stimulate an immediate behavioral response in the pheromone recipient, or as primers if they do not induce a behavioral response but result in a physiological change in the receiver. In general, physiological responses to primer pheromones are thought to occur only after a delay of hours or days. However, although these terms are useful in indicating the speed at which pheromone-stimulated sexual responses occur, the distinction between releaser and primer pheromones does not necessarily indicate differences in the nature or initial mode of action of the pheromones themselves (Stacey et al.,1986).

Examples of releaser and primer sex pheromones are wide-spread in nature (Birch, 1974; Birch and Haynes, 1982; Shorey, 1976; Free, 1987). In general, studies on insects

have provided the greatest information on pheromone identity and function. For example, female butterflies and moths produce releaser pheromones which attract males over great distances (Birch and Haynes, 1982). An example of an insect primer pheromone is provided by the queen bee which releases a pheromone that inhibits ovarian development in worker bees (Free, 1987).

The chemical composition of many insect pheromones has been identified through a variety of biochemical techniques (Birch and Haynes, 1982); the most stimulatory compounds appear to be aldehydes, alcohols and/or acetates which are highly volatile in the terrestrial environment (Tumlinson and Teal, 1987). In most cases, females produce and release the species specific sex attractants, although males may also produce and release pheromonal substances. Both sexes have evolved specialized sensory cells on their antennae which allow them to detect pheromones. Changes in the electrical activity of the sensory cells following pheromone delivery can be recorded electrophysiologically in the form of electroantennograms (EAGs: Birch and Haynes, 1982; De Kramer and Hemberger, 1987). Both the EAG technique and the development of behavioral bioassays have allowed researchers to examine structure-activity relationships of both naturally occurring and synthetic compounds which exert pheromonal actions in a variety of insects (Birch and Haynes, 1982; Free, 1987). Although insect pheromones are considered to be "simple" in that they are comprised of a few chemicals whose composition is well defined and normally consistent within a species, the nature of insect pheromones is extremely complex, since it is becoming increasingly evident that a proper blend of chemicals is responsible for evoking biological responses in conspecifics (Birch and Haynes, 1982).

Releaser and primer pheromones also are involved in mammalian reproduction. Virtually all mammals which have been tested have shown the ability to discriminate males from females by their odor alone. A familiar example is that of the behavioral response of a male dog to the odor of a bitch in heat. The primary source of mammalian sex pheromones appears to be from the urine or vaginal secretions, although specialized scent glands (anal and skin), feces and saliva contribute to the delivery of a variety of pheromones to conspecifics (Birch, 1974; Shorey, 1976).

Most mammals investigate and nuzzle the genitalia of their sexual partners during courtship, often leading to stereotyped behavioral responses. For example, the vaginal discharge of female hamsters contains a pheromone that exerts potent releaser effects on male behavior; males will attempt to copulate with objects and/or other males scented with hamster vaginal secretions (Springer et al., 1980). Primer effects of sex pheromones in mammalian reproduction have also been well documented (Muller-Schwartze and Silverstein, 1980). Most, if not all, of the putative primer sex pheromones are believed to operate through neuroendocrine mechanisms to affect reproduction (Bronson, 1982). These include pheromones in the urine of male mice which delay puberty in juvenile males but accelerate puberty in juvenile females (the *Vandenbergh effect*, Vanderburgh, 1967, 1969), odors that induce estrus and ovulation in previously anestrus adult females (the *Whitten effect*, Whitten and Champlin, 1972), or those that cause failure of embryo implantation and the re-initiation of the estrus cycle in recently mated females (the *Bruce effect*, Bruce, 1960; Bruce and Parrot, 1960). In addition, urine from females mice can induce a rapid surge of luteinizing hormone (LH) in adult males whereas the odor of a dominant male may suppress levels of LH in subordinate males (Bronson, 1982).

In contrast to the situation in insects, the understanding of sex pheromone function in mammals has been impeded by a lack of information on chemical identities. In most cases, attempts at fractionating the substances (urine and vaginal secretions) which contain biological activity have failed to identify the chemical nature of the pheromone(s) in question. For example, the vaginal discharge of female hamsters stimulates male copulatory behavior (ie. mounting and pelvic thrusting related to intromission), and also attracts males to females, decreases male aggressive behavior, increases male testosterone, and results in a reduction of male scent marking (Springer et al, 1982). However, it is unclear if each response is elicited by different compounds, or if various mixtures of compounds are involved (Springer et al., 1982). Volatile compounds in the vaginal discharge have been collected following distillation and gas chromatography and tested in a behavioral assay which allowed male hamsters to dig, through the cage bedding, to gain access to the odor source (Springer et al., 1982). The fractions most active in eliciting attraction of males were

found to contain appreciable amounts of dimethyl disulfide. However, authentic dimethyl disulfide was found to be only half as effective as fresh vaginal discharge when tested in the bioassay. The difference between the attractant activity of fresh discharge and dimethyl disulfide does not appear to be due to the presence of any single additional volatile compound, since gas chromatographic fractions containing only one other compound, besides dimethyl disulfide, were ineffective at eliciting normal behavioral responses from males. In addition, mixtures of all other identified volatile compounds (acids and alcohols) were also inactive in the attractant assay and only 60% of normal activity was obtained with a grand mixture of acids, alcohols and dimethyl disulfide (Springer et al., 1982). Although dimethyl disulfide appears to be an important component of the attractant pheromone, it is clear that other unidentified compounds contribute to the attractive quality of the vaginal discharge. Similar attempts have been made to identify the mounting pheromone(s) in hamster vaginal discharge (Springer et al., 1982, 1986).

The attempts at identifying the chemical structure of the puberty-accelerating primer pheromone from male mouse urine have been less successful than those associated with identifying hamster releaser pheromones. In general, partial purifications indicate that the puberty-accelerating pheromone is associated with a relatively non-volatile protein fraction of urine (Novotny et al., 1982), although volatile substances may also be involved (Jemiolo et al., 1986). These studies raise important questions as to whether specific volatile compounds function as pheromones in mammals or whether the pheromones are mixtures of volatile and non-volatile compounds which create a bouquet of odors that elicit specific behavioral and physiological responses in conspecifics.

The involvement of sex pheromones in teleost reproduction has been extensively reviewed in recent years (Colombo et al., 1982; Liley, 1982; Liley and Stacey, 1983; Stacey et al., 1986; Resink, 1988). Releaser pheromones that evoke attraction and/or reproductive behavior of the opposite sex have been described in a wide variety of teleosts (see review: Stacey et al., 1986). The available evidence suggests that both male and female fish use sex pheromones as specific signals for the onset of spawning activity. For

example, in females, the production and release of behavioral releasing pheromones appear to be restricted to, or maximal during, the period shortly after ovulation (Stacey et al., 1986). Since most female fish are sexually active only during this period, the putative pheromones appear to synchronize male sexual behavior with female spawning readiness. Similarly, males release pheromones to attract females (Stacey et al., 1986), and in some cases to attract ovulated, but not unovulated females (Lee and Ingersoll, 1979). Reports on primer effects of sex pheromones in teleosts are less plentiful in the literature. However, male sex pheromones are believed to stimulate ovarian growth and ovulation in zebrafish, *Brachydanio rerio* (Van den Hurk et al., 1987), angelfish, *Pterophyllum scalare* (Chien, 1979) and African catfish, *Clarias gariepinus* (Resink et al., 1988) and spermiation in goldfish, *Carassius auratus* (Stacey and Sorensen, 1986).

Despite the considerable observational and experimental evidence for the existence of sex pheromones in fish, there is relatively little known about the production, release or chemical identity of teleost sex pheromones. In the majority of species examined, sex pheromones appear to be of gonadal origin, although skin, secondary sex structures, and urine also have been implicated (Colombo et al 1982; Liley, 1982). Unlike the highly volatile insect and/or proteinaceous mammalian pheromones (Springer et al., 1986), recent evidence suggests that teleosts use reproductive hormones and their metabolites as sex pheromones (Sorensen and Stacey, 1989). More specifically, prostaglandins (PGs) and a variety of gonadal steroids (i.e. androgens, estrogens and progestogens) have been shown to function as sex pheromones in a number of fish. For example, water-borne steroid hormones or their conjugated metabolites (i.e. glucuronides) appear to be responsible for the attraction of female black goby, *Gobius jozo* (Colombo et al., 1980, 1982), male zebrafish (Van den Hurk and Lambert, 1983) and male guppy, *Poecilia reticulata* (Johansen, 1985), and for inducing ovulation in zebrafish (Van den Hurk et al., 1987) and African catfish (Resink, 1988), and spermiation in goldfish (Stacey and Sorensen, 1986). Moreover, exposure of males to water-borne PGs and/or PG-injected females has been shown to stimulate male courtship and spawning behavior in goldfish (Sorensen et al., 1986; 1988), fathead minnows, *Pimphales promelas* (Cole and Smith, 1987), and

milkfish, *Chanos chanos* (Kelly, Tamaru and Lee, personal communication). Since PGs have been directly implicated in the control of follicular rupture at the time of ovulation, and the activation of female spawning behavior in some teleosts (Stacey and Goetz, 1982), PGs appear to serve as good indicators of female reproductive condition following ovulation. The same argument has been made for gonadal steroids which function endogenously to mediate a variety of reproductive functions prior to being released to the water as sex pheromones (Sorensen and Stacey, 1989).

Sorensen and Stacey (1989) have extended earlier theories (Doving, 1976; Colombo et al., 1982) to explain why fish have evolved the use of hormones and their metabolites as pheromones: 1) hormones and their metabolites represent pre-existing signals produced in temporal synchrony with discrete reproductive events; 2) hormones are readily excreted to the water; and 3) the evolution of olfactory receptors for water-borne hormones may have resulted from prior existence of internal endocrine receptors. In addition, species-specificity of pheromone use in teleosts may be achieved by using different mixtures of hormones, which may include non-hormonal components, or by modifying hormonal metabolic pathways for the production of hormonal pheromones (Sorensen and Stacey, 1989). These arguments also apply to situations in invertebrates (crab: Kitteredge et al., 1971) and vertebrates (newt: Belvedere et al., 1988; pig: Sink, 1967) where released steroid hormones and steroid metabolites have been shown to exert pheromonal actions.

The most comprehensive studies which link hormones to pheromone function in fish have been conducted on goldfish (see review Sorensen and Stacey, 1989). In temperate climates, female goldfish ovulate in the spring in response to rising water temperatures and emerging aquatic vegetation (Stacey et al., 1979). Females ovulate in the early morning when light levels are low, and like other oviparous teleosts, become sexually active at the time of ovulation (Stacey, 1987). Since females spawn their eggs within a few hours of ovulation, male-female reproductive physiology and behavior must be synchronized to ensure reproductive success. This synchrony is achieved by the release of both preovulatory primer and postovulatory releaser pheromones by females which affect male reproductive physiology and behavior, respectively (Sorensen and Stacey, 1989). The

substances which are believed to comprise each of these pheromones are known to function as reproductive hormones in females (see below). In addition, the production and release of both pheromones are closely associated with the occurrence of a preovulatory surge of blood gonadotropin (GtH) which begins in the latter half of the photophase and induces ovulation, approximately 12 h later, in the latter half of the scotophase (Stacey et al., 1979).

Considerable evidence suggests that the preovulatory primer pheromone in goldfish is a gonadal steroid. For example, approximately 10 h prior to ovulation, female goldfish synthesize increased quantities of the steroid hormone, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), which induces final maturation of developing oocytes (Goetz, 1983). 17,20B-P is then released to the water (Stacey et al., 1989) where it functions as a potent olfactory stimulant (Sorensen et al., 1987). Males exposed to water-borne 17,20B-P have increased milt (sperm and seminal fluid) volume the following day (Stacey and Sorensen, 1986).

The experiments described in this thesis examined the time-course and central regulation of sperm production in response to water-borne 17,20B-P in goldfish. Chapter 2 presents evidence that the milt response to the pheromone is mediated by an acute rise in blood GtH in males. The involvement of olfaction and the olfactory pathways by which water-borne 17,20B-P stimulates GtH release and milt production in goldfish was determined by examining the effects of various olfactory tract lesions on these 17,20B-P-induced responses (Chapter 3). In Chapter 4, the olfactory sensitivity of male goldfish to water-borne 17,20B-P was studied by determining the threshold water concentration of 17,20B-P required to induce GtH release in males. In Chapter 5, the time-course and specificity of male endocrine responses to water-borne 17,20B-P were examined by measuring changes both in serum levels of GtH, growth hormone (GH), testosterone (T), and 17,20B-P (of testicular origin), and in milt volume, following extended (8-12 h) exposure to the pheromone. Possible day-night differences in male endocrine responsiveness to water-borne 17,20B-P were examined in Chapter 6. In Chapter 7,

associated changes in GtH and GH secretion in males exposed to either 17,20B-P-releasing or PG-releasing females were compared to those responses observed following exposure of males to 17,20B-P and PGs alone. In Chapter 8, the neuroendocrine regulation of the 17,20B-P-induced GtH response in male goldfish was examined. Since dopamine (DA) inhibits, while gonadotropin-releasing hormone (GnRH) stimulates GtH release in goldfish (Peter et al., 1986), possible changes in brain and pituitary levels of these substances were monitored in males exposed to water-borne 17,20B-P. Finally, Chapter 9 summarizes and discusses the major findings of the thesis and provides the basis for a model to study primer sex pheromone function in teleosts as well as other vertebrates.

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2. A STEROID SEX PHEROMONE SYNCHRONIZES MALE-FEMALE SPAWNING READINESS IN GOLDFISH [1]

2.1 INTRODUCTION

Understanding of pheromone function in teleost fish has been impeded by a lack of pheromone identities (Stacey et al., 1986). However, recent studies (Stacey and Sorensen, 1986; Sorensen et al., 1987) on goldfish, *Carassius auratus*, provide strong evidence that 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), the proposed oocyte maturation-inducing steroid hormone in goldfish (Nagahama et al., 1983) and other teleosts (Goetz, 1983), could be a potent female sex pheromone. Milt (sperm and seminal fluid) volume in goldfish is increased by exposure to 17,20B-P, and to a lesser extent by exposure to two precursors of 17,20B-P (progesterone and 17α-hydroxyprogesterone), but not to other steroids proposed as fish pheromones (Stacey et al., 1986; Colombo et al., 1982; Van den Hurk and Lambert, 1982). In addition, the goldfish olfactory epithelium is extremely sensitive to 17,20B-P (Sorensen et al., 1987), and the increase in milt volume normally induced by 17,20B-P exposure is abolished by sectioning the medial olfactory tracts, which previously have been implicated in the control of sex behavior in male goldfish (Stacey and Kyle, 1983). I report here that ovulating goldfish release 17,20B-P into the water and that a rapid (within 15 min) elevation in blood gonadotropin (GtH) of males mediates the milt response to 17,20B-P exposure. I conclude that this pheromone system synchronizes milt production with ovulation.

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2.2 MATERIALS AND METHODS

Animals

Sexually mature male and gravid female goldfish of the common comet variety were purchased from Ozark Fisheries, Stoutland, Missouri. Stocks of separate sexes were kept in 1000 L flow-through aquaria at 14-20° C under a 16L:8D photoperiod. Fish were fed *ad libitum* with Nutrafin flaked food or Ewos pellets at least once a day.

Induction of Ovulation

The goldfish is well suited for studies of pheromone release by females and pheromonal effects on males because ovulation is synchronized with the light-dark cycle and can be manipulated under laboratory conditions. Goldfish which have completed vitellogenesis at 12-14° C ovulate spontaneously within a day of being warmed to 20° C and exposed to aquatic vegetation, the substrate for oviposition. On a 16 hours light: 8 hours dark photoperiod, the preovulatory GtH surge begins after the midpoint of the photophase and induces ovulation (follicular rupture) about 10-12 hours later, during the latter half of the scotophase (Stacey et al., 1979); oocyte final maturation is completed halfway between the initiation of the GtH increase and ovulation (Stacey et al., 1984). Spawning begins shortly after ovulation.

Experiment 1.

To determine whether females release 17,20B-P, six mature females were maintained individually in 1 L of water for four successive four-hour intervals encompassing the periovulatory period. At 21:00 h on day 1, the females were transferred from stock tanks (14° C) to 65 L aquaria (20° C; 16L:8D) containing artificial vegetation. At 19:00 h on day 2, when the preovulatory surge of GtH (Stacey et al., 1979) should have commenced, females were removed and placed individually in glass jars containing 1 L of aerated water at 20° C. At 23:00 h, a 10 ml water sample was taken from each jar for 17,20B-P determination and each female was checked for ovulation and transferred to a clean jar.

This procedure was repeated four times, the last water sample being taken at 11:00 h on day 3. Water samples were extracted two times with 2 vol ether and the amount of 17,20B-P in the extract determined by radioimmunoassay (RIA; Scott et al., 1982; Van Der Kraak et al., 1984).

Experiment 2.

I next used hypophysectomized males to determine whether exposure to 17,20B-P increases milt volume by stimulating the hypothalamic-pituitary-gonadal axis. On day 1, males were anesthetized (0.1% 2-phenoxyethanol, Syndel), then either hypophysectomized by the opercular approach (Yamazaki, 1965), sham operated, or handled without surgery, and placed by groups in 65 L flow-through aquaria (four fish per aquarium) at 20° C. At 17:00 h on day 3, fish were stripped of milt to determine initial milt levels. The fish were placed belly-up in a slotted foam pad and gentle pressure was applied to the abdomen to express milt into weighed (±0.1 mg) haematocrit tubes with weighed caps which were then weighed to determine the weight of milt. Milt density was assumed to be 1.0 and milt values are expressed as volumes (µl) rather as weights. At 21:00 h, the water flow to all aquaria was shut off and 10 µg 17,20B-P (Sigma) in 0.1 ml of ethanol was added to each aquarium; this dose should have produced a water concentration of approximately 150 pg/ml ($5X10^{-10}$ M), although actual water concentrations were not measured. At 09:00 h on day 4, all fish were again anesthetized and stripped of milt to determine milt volumes following exposure to 17,20B-P. Within each group, milt volumes before and after exposure to 17,20B-P were compared by the Wilcoxon matched-pairs signed-ranks test.

Experiment 3.

I next determined whether GtH, known to increase milt production when injected into a variety of teleosts including goldfish (Billard et al., 1982), increases in the blood of males exposed to water-borne 17,20B-P. I also measured serum 17,20B-P levels as 17,20B-P of testicular origin has been shown to mediate the action of GtH on milt production (Ueda et al., 1985). On day 1, males were placed in 65 L flow-through aquaria (four fish per

aquarium) at 20° C. During scotophase on day 2, 10 µg 17,20B-P in 0.1 ml ethanol was added to each experimental aquarium and blood samples were taken from different groups of males under anesthesia 15, 30, 60, and 120 min later; control males were bled following 60 min exposure to ethanol vehicle. All blood samples were taken during the scotophase (02:00-06:00 h) and all treatment groups were balanced over time. Following blood sampling, all fish were transferred to clean flow-through aquaria and milt sampled 7-8 h after exposure to 17,20B-P had begun. Serum GtH and 17,20B-P concentrations were determined by RIA (Peter et al., 1984; Scott et al., 1982; Van Der Kraak et al., 1984). Data on GtH and 17,20B-P were analysed by ANOVA and Newman-Keuls procedure. Milt volume data were analysed by Kruskal-Wallis ANOVA and Mann-Whitney U tests.

Experiment 4.

A pheromone-mediated increase in milt volume could affect reproductive success if the increase occurs prior to ovulation. I therefore determined the latency of the 17,20B-P-induced milt response. Males were placed in 65 L flow-through aquaria (three fish per aquarium) at 20° C. At the onset of the scotophase, either 0.1 ml of ethanol or 10 µg 17,20B-P in 0.1 ml ethanol was added to each aquarium. Three and six hours later, different groups of males were anesthetized for blood sampling and determination of milt volume. Data on GtH were analysed by *t*-tests and milt volume data were analysed by Mann-Whitney U tests.

2.3 RESULTS

Experiment 1.

Three of the six females ovulated in the third four-hour interval and released high levels of 17,20B-P into the water during the first and second four-hour intervals (Fig. 2.1). All water samples from the females which did not ovulate had relatively low concentrations

of 17,20B-P.

Experiment 2.

When hypophysectomized males were stripped of milt two days after surgery and then exposed to a water concentration of 17,20B-P ($5X10^{-10}$ M) approximating the peak concentrations released by ovulating females (see Fig. 2.1), milt volumes which could be stripped the following morning were significantly ($p<0.001$) reduced (Fig. 2.2). In contrast, milt volumes of males which had undergone a sham operation and of intact males were significantly ($p<0.001$) increased following this exposure to 17,20B-P.

Experiment 3.

Experimental males exposed during the scotophase to 17,20B-P ($5X10^{-10}$ M) for 15, 30, 60, or 120 min had significantly ($p<0.01$) higher GtH concentrations than did control males exposed to the ethanol vehicle for 60 min (Fig. 2.3A). As well, milt volumes stripped 7-8 h after 17,20B-P exposure were significantly ($p<0.01$) greater in the experimental groups than in the control group (Fig 2.3C). Consistent with the hypothesis that endogenous 17,20B-P mediates GtH-induced milt increases in goldfish, serum 17,20B-P levels in three of the four groups exposed to water-borne 17,20B-P were significantly ($p<0.01$) higher than in control males exposed to ethanol (Fig. 2.3B). It is unlikely that passive uptake of 17,20B-P from the aquarium water accounts for these differences between experimental and control groups because serum 17,20B-P levels in all groups were higher than the calculated water concentration of 17,20B-P.

Experiment 4.

Serum GtH levels in males exposed to water-borne 17,20B-P at 3 and 6 h were significantly higher ($p<0.01$ and $p<0.05$, respectively) than those of control males (Fig. 2.4A). However, only at the 6 h sample time were the milt volumes of males exposed to 17,20B-P greater ($p<0.05$) than those of controls (Fig. 2.4B). As peak preovulatory release of 17,20B-P from females occurs more than 4 h prior to ovulation (see: Fig. 2.1),

the milt response to water-borne 17,20B-P appears to be rapid enough to allow an increase in milt production prior to spawning.

2.4 DISCUSSION

The results presented in this and our earlier studies (Stacey and Sorensen, 1986; Sorensen et al., 1987) clearly show that 17,20B-P, which promotes oocyte final maturation in goldfish (Nagahama et al., 1983) and is released from females prior to ovulation, also increases blood GtH and milt volume in males. Indirect evidence that these physiological responses are normally stimulated by females is provided by a recent observation (Kobayashi et al., 1986) that the presence of an ovulating female goldfish induces a GtH surge in males coincident with that of the female. Both the temporal pattern of 17,20B-P release from preovulatory females and the latency of the milt response to water-borne 17,20B-P support our proposal that 17,20B-P is a pheromone which synchronizes milt production with ovulation. This appears to be the only vertebrate system in which the identity, cellular source and release of the pheromone, as well as the sensory pathway, endocrine mediators and the significance of the response in the pheromone recipient, all are clearly established.

Since this publication, a subsequent investigation has provided direct evidence that 17,20B-P functions as a female sex pheromone in goldfish. In this study (Stacey et al., 1989), we more accurately determined periovulatory patterns of endogenous production and release of 17,20B-P and examined the associated male endocrine responses which occur during exposure to 17,20B-P-releasing females. This paper is included in the appendix section of the thesis to give the reader a more accurate understanding of the 17,20B-P-pheromone system in goldfish (see appendix II: Stacey et al., 1989).

Figure 2.1. Concentrations of 17,20B-P in female holding water during the periovulatory period.

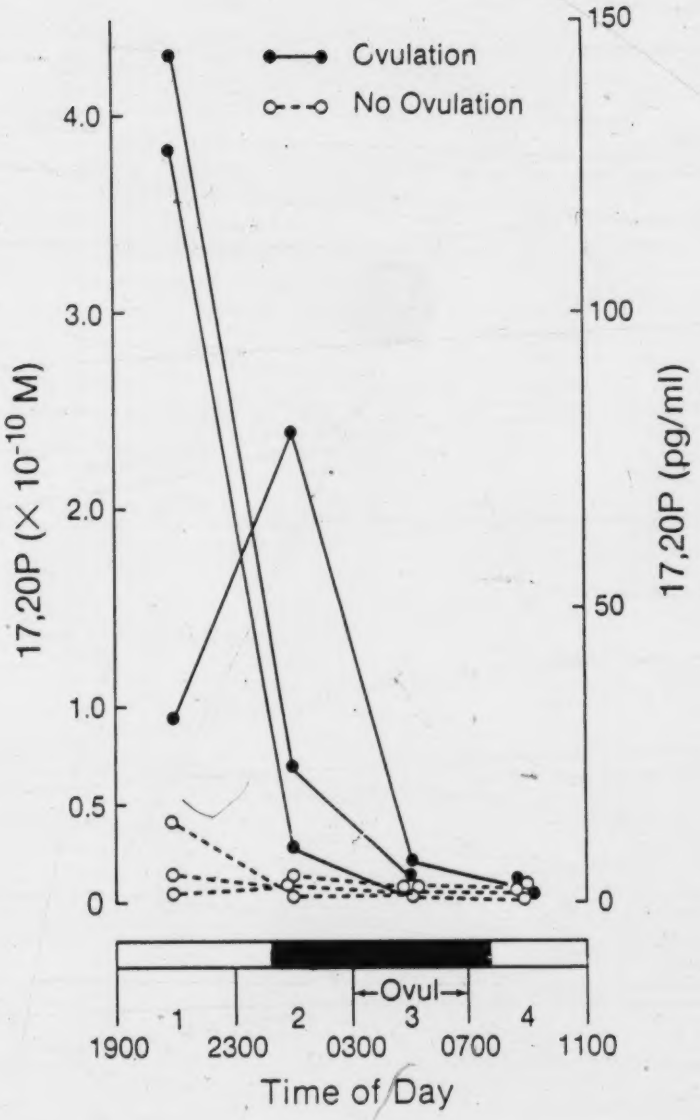

Ovulation
No Ovulation
17,20P (× 10⁻¹⁰ M)
17,20P (pg/ml)
4.0
3.0
2.0
1.0
0.5
0
150
100
50
0
Ovul
1
2
3
4
1900
2300
0300
0700
1100
Time of Day

Figure 2.2. Effect of hypophysectomy on the 17,20B-P-induced increase in milt volume in male goldfish. Open bars, initial milt volume; shaded bars, milt volume after exposure to 17,20B-P. Males were hypophysectomized (Hypox), had undergone a sham operation (Sham) or were intact (Intact); ***, significantly different from pre-exposure values ($p<0.001$); n=20 in all groups.

Median milt volume (ul)
100
80
60
40
20
0
***
***
***
Intact
Sham
Hypox.

Figure 2.3. Effect of water-borne 17,20B-P on serum GtH (**A**), serum 17,20B-P (**B**), and (**C**) milt volume in male goldfish. Values in **A** and **B** are means; lines, ±s.e.m.; **, significantly different from controls ($p<0.01$). Numbers within bars show sample size.

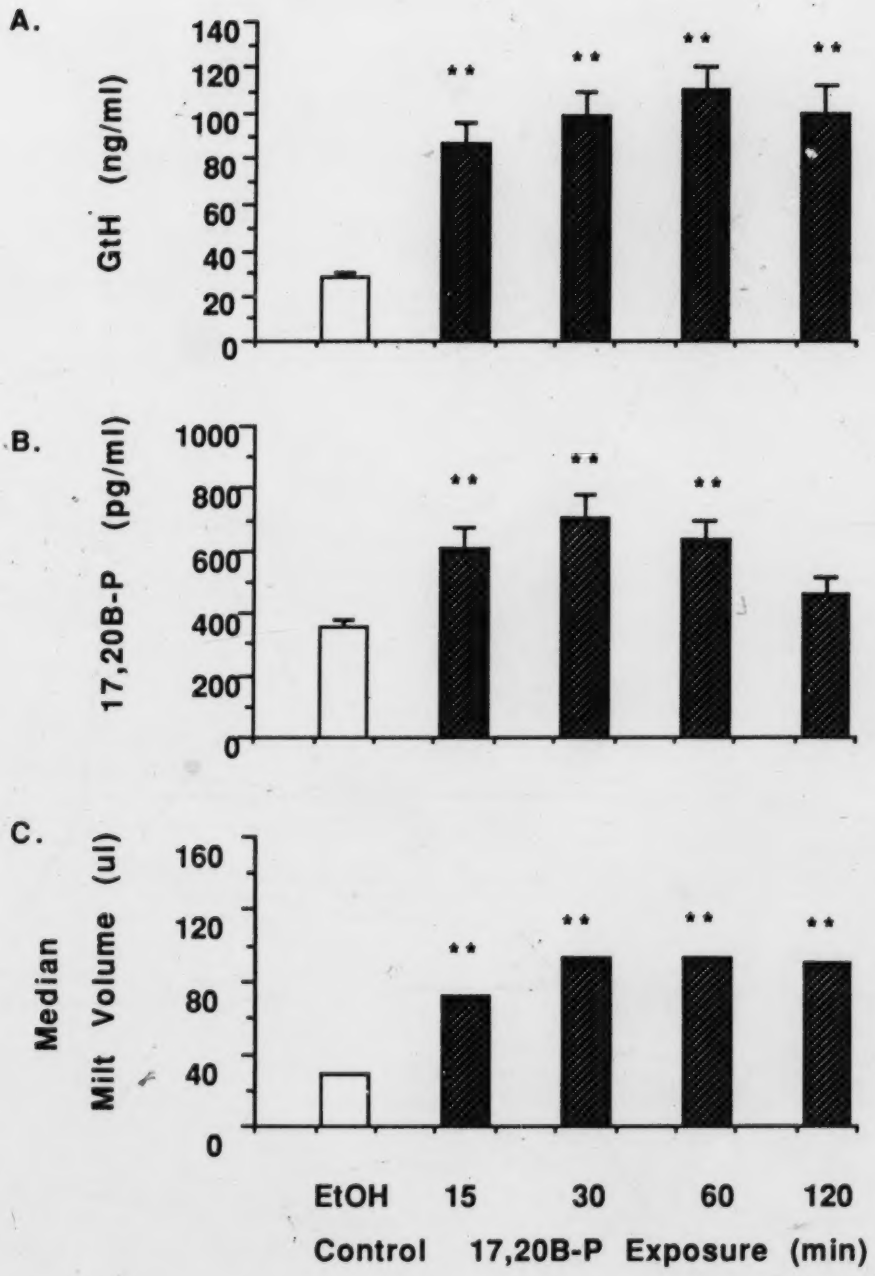
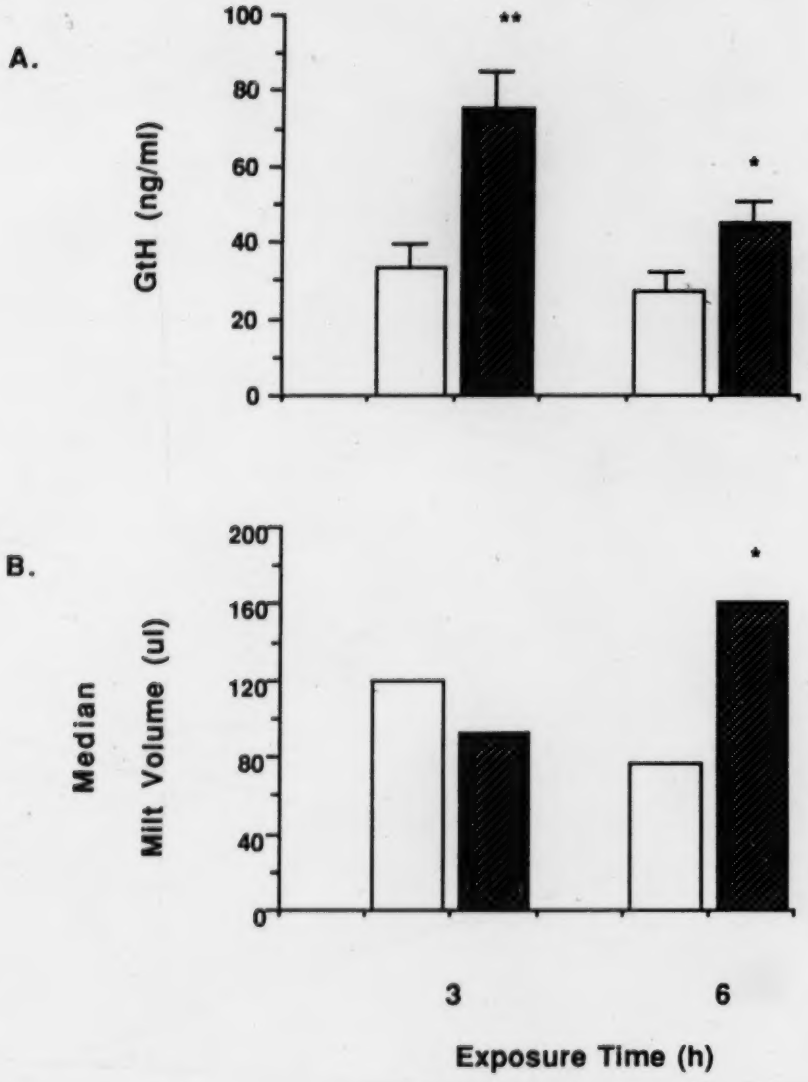

A.
GtH (ng/ml)
140
120
100
80
60
40
20
0
**
B.
17,20B-P (pg/ml)
1000
800
600
400
200
0
C.
Median Milt Volume (ul)
160
120
80
40
0
EtOH
15
30
60
120
Control
17,20B-P Exposure (min)

Figure 2.4. Effect of 3 and 6 hours of exposure to water-borne 17,20B-P on serum GtH (**A**) and milt volume (**B**) in male goldfish. Values in **A** are means; lines, s.e.m.; **, $p<0.01$; *, $p<0.05$ (significantly different from controls); n=15 in all groups.


A.
GtH (ng/ml)
100
80
60
40
20
0
**
*
B.
Median
Milt Volume (ul)
200
160
120
80
40
0
*
3
6
Exposure Time (h)

2.5 LITERATURE CITED

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3. EFFECTS OF OLFACTORY TRACT LESIONS ON GONADOTROPIN AND MILT RESPONSES TO THE FEMALE SEX PHEROMONE, 17α,20B-DIHYDROXY-4-PREGNEN-3-ONE, IN MALE GOLDFISH

3.1 INTRODUCTION

In goldfish, the gonadal steroid, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), acts as a potent female sex pheromone which synchronizes milt production with ovulation (Stacey and Sorensen, 1986; Dulka et al., 1987; Stacey et al., 1989). This steroid, which functions endogenously in females to promote oocyte final maturation (Nagahama et al., 1983), is released to the water as a preovulatory surge which commences approximately 8-10 h prior to ovulation and declines dramatically at the time of ovulation (Dulka et al., 1987; Van Der Kraak et al 1989; Stacey et al., 1989). Electrical recordings from the olfactory epithelium demonstrate that males are extremely sensitive to low water concentrations of 17,20B-P (olfactory threshold, 10^{-13}-10^{-12} M; Sorensen et al., 1987a; Sorensen et al., 1987b). Detection of the pheromone by males leads to increases in gonadotropin (GtH) secretion within 15 min and milt production within 6 h (Dulka et al., 1987). In addition, a recent investigation has demonstrated a close temporal relationship between the release of 17,20B-P by preovulatory females and increased GtH levels and milt volume in males (Stacey et al., 1989).

As with pheromonal responses in many other teleosts (Stacey et al., 1986; Kyle et al., 1987; Resink, 1989), the 17,20B-P-induced milt response in male goldfish is dependent on the olfactory system (Stacey and Sorensen, 1986). Goldfish possess pedunculated olfactory bulbs which receive olfactory information from the olfactory epithelium and are connected to the telencephalon by long olfactory tracts (OTs). Each OT consists of a medial olfactory tract (MOT) and a lateral olfactory tract (LOT) which give rise to predominantly separate terminal fields within the forebrain (Von Barthe!d et al., 1984;

Levine and Dethier, 1985, Springer, 1983). Stacey and Sorensen (1986) demonstrated that the 17,20B-P-induced milt response in goldfish is abolished by bilateral OT section, but not by unilateral OT section. Furthermore, in unilateral OT-sectioned males, the milt response was abolished by sectioning the contralateral MOT, but not the contralateral LOT. However, since the GtH response to 17,20B-P had not yet been identified, Stacey and Sorensen (1986) did not examine the effects of OT lesions on GtH release. This study sought to determine whether OT section blocks the 17,20B-P-induced GtH increase in male goldfish, and whether the integrity of the MOTs is necessary for the response to occur.

3.2 MATERIALS AND METHODS

Animals

Goldfish of the common comet variety (20-30 g) were purchased from Ozark Fisheries Co. Stoutland Missouri and kept in all-male groups in 1000 L flow-through stock aquaria at 18-20° C under a 16L:8D photoperiod. Fish were fed Ewos trout pellets or Nutrafin flaked food *ad libitum* at least once a day. Only spermiated males with well developed pectoral fin tubercles were used in the experiment.

Surgical procedure

The experiment was conducted in February and March 1987. On day 1, groups of males were removed from stock aquaria, anesthetized in a 0.05% solution of 2-phenoxyethanol (Syndel), fin clipped for identification and given one of five treatments as described in Stacey and Kyle (1983) and Stacey and Sorensen (1986). Control fish (INTACT), receiving no surgical operation, were revived and transferred to 65 L flow-through aquaria at 20° C. In the remaining groups of males, the paired OTs were exposed by cutting a 3-sided bone flap through the cranium with a dental saw. The overlying adipose tissue and cranial fluid were removed by gentle aspiration. This procedure normally exposed the entire length of the OTs extending from the rostrally situated

olfactory bulbs to the anterior pole of the telencephalic hemispheres. SHAM operated males received no further treatment to the OTs; the cranial cavity was filled with teleost saline and the bone flap was returned to its normal position by gently pushing its free margin beneath the edge of the skull. The remaining 3 groups of males received bilateral surgical operations to the paired OTs. In two of these groups, the lateral and medial sub-divisions of each OT were separated longitudinally using the tip of a 25 gauge syringe needle. In one group, only the lateral components of the paired OTs were severed (LOTX), leaving the medial sub-divisions intact. In the other group, the medial sub-divisions were cut (MOTX), leaving the lateral components intact. Finally, one group of males received total OT section (OTX) by severing both OTs completely. After the operations, fish in each treatment group were revived and placed in groups of 3 in 65 L test aquaria and allowed to recover for 2 days.

Experimental Procedure

Stock solutions of 17,20B-P (Sigma) were prepared by dissolving the steroid in 98% ethanol (EtOH, 1.0 mg/ml). Working solutions of 17,20B-P were further diluted (1:10) with EtOH for use in the experiment. At 09:00 hrs on day 3, the water flow to the test aquaria was shut off. Half of each treatment group was exposed to 17,20B-P at a dose of 10 µg/100 µl EtOH which was added directly to the aquaria water using a glass syringe, The amount of 17,20B-P added to each aquarium should have resulted in a final tank concentration of approximately $5X10^{-10}$ M, although actual water concentrations were not determined. The remaining half of each group of males was exposed to 100 ul of EtOH (Control). Following 1 h of exposure to either 17,20B-P or EtOH, all fish were anesthetized, blood sampled through the caudal vasculature, and returned to their respective aquaria. On the morning of day 4, the amount of milt which could be stripped from each fish was collected in pre-weighed hematocrit tubes which were then fitted with weighed caps as described in Stacey and Sorensen (1986). The filled tubes were then weighed and the weight of the milt calculated. Assuming a milt density of 1.0, the milt values are expressed as volumes (µl) instead of weights.

Following milt sampling, each surgical group was combined (6 fish/aquarium) in flow-through aquaria until being exposed to 17,20B-P or EtOH for a second time one week later. On day 9, fish from each surgical group were randomized, stripped of milt to standardize pre-exposure milt levels and then placed in groups of 3 in test aquaria. On day 10, the water flow to all aquaria was shut off and the exposure procedure was repeated with one half of each surgical group being exposed to 17,20B-P and the other half being exposed to EtOH. Blood samples were taken 1 h after exposure and milt was again stripped from all fish the morning of day 11, as described above. The experiment was carried out in two runs, three days apart, each consisting of one half of the total sample size (n=10-12) for each treatment group.

Hormone Assays

All blood samples were allowed to clot for at least 4 h on chipped ice and the serum from each sample was collected and stored at -25^{o} C until assayed for GtH by radioimmumoassay (RIA) as described previously (Peter et al., 1984).

Statistical Analysis

GtH and milt values were log transformed and analysed with a two-way analysis of variance (ANOVA) to compare differences between surgical treatments. For each surgical treatment, differences between responses to EtOH and 17,20B-P were determined by Least Squares Means method using a SAS computer package (Barr et al., 1979).

3.3 RESULTS

Two days post-OT lesion

Blood GtH levels in INTACT and LOTX males exposed to EtOH were similar but significantly ($p<0.01$) higher than MOTX and OTX groups (Fig. 3.1A). The GtH levels in SHAM males exposed to EtOH were similar to those of INTACT males, but significantly

($p<0.01$) higher than all other groups exposed to EtOH (Fig. 3.1A). Compared to the groups exposed to EtOH, 17,20B-P significantly increased plasma GtH in INTACT ($p<0.05$) and LOTX ($p<0.01$) males, but failed to evoke a GtH response in MOTX and OTX males. SHAM males exposed to 17,20B-P had elevated levels of GtH which were statistically similar to those of INTACT and LOTX males exposed to 17,20B-P. However, the GtH levels in 17,20B-P-exposed SHAM males were not significantly different from those exposed to EtOH, which showed unusually high levels of GtH.

The treatment effects on milt volume were identical to those on GtH (Fig. 3.1B). INTACT and SHAM operated males exposed to EtOH had significantly ($p<0.01$) higher milt volumes than all other groups exposed to EtOH. Milt volume was significantly elevated in INTACT ($p<0.05$) and LOTX ($p<0.01$) males exposed to 17,20B-P compared to EtOH-exposed controls. SHAM males exposed to EtOH and 17,20B-P had similar volumes of milt.

One week post-OT lesion

There were no differences in GtH levels among groups exposed to EtOH (Fig. 3.2A). Exposure to 17,20B-P significantly ($p<0.001$) increased GtH in INTACT, SHAM and LOTX males. MOTX and OTX treatments abolished the GtH responses to 17,20B-P.

Pre-exposure milt volumes were similar in all groups ($p>0.05$, 2-way ANOVA; data not shown). The milt volume of the SHAM group exposed to EtOH was significantly ($p<0.05$) greater than LOTX, MOTX and OTX groups, but similar to that of INTACT males. INTACT and LOTX males exposed to 17,20B-P had significantly ($p<0.001$) elevated volumes of milt compared to EtOH exposed controls (Fig. 3.2B). The difference in milt volume between SHAM fish exposed to 17,20B-P and EtOH approached significance ($p=0.058$).

3.4 DISCUSSION

The results of this study support the earlier finding that lesions of the MOTs abolish the milt response to water-borne 17,20B-P (Stacey and Sorensen, 1986) and provide additional evidence that the pheromone-induced increase in milt production is mediated by elevated GtH release in male goldfish. Selective sectioning of the MOTs, but not the LOTs, significantly blocked both the GtH and milt responses to 17,20B-P. Furthermore, LOTX fish, which had intact MOTs, exhibited pheromone-induced increases in GtH release and milt production which were similar to INTACT and SHAM males. The confirmation that both the GtH and milt responses to 17,20B-P are dependent on the MOTs supports the hypothesis that this olfactory subdivision mediates reproductive responses to pheromones in goldfish (Kyle et al., 1987). In addition, the present results, and the recent finding that hypophysectomy blocks the milt response to 17,20B-P (Dulka et al., 1987), provide further evidence that the milt response to 17,20B-P is triggered by the activation of the hypothalamic-pituitary-gonadal axis. Elevated levels of plasma GtH are believed to act through testicular mechanisms to promote increased milt production. In this regard, the 17,20B-P-induced GtH responses in male goldfish are accompanied by increases in blood 17,20B-P (Dulka et al., 1987). This observation is consistent with the proposal that testicular 17,20B-P mediates the action of GtH on milt production in goldfish and some other teleosts (Ueda et al., 1985).

Except for the SHAM animals tested two days after surgery, the effects of the various OT lesions were consistent over the two testing periods. It is not known why SHAM males exposed to EtOH two days after surgery had high levels of plasma GtH. The increased levels of GtH in these animals undoubtedly contributed to their high milt volumes. If short-term post-operative stress effects were responsible for these abnormally high GtH and milt levels, they were greatly reduced 1 week later.

Results from anatomical, behavioral and electrophysiological studies suggest that the MOTs, and not the LOTs, are primarily involved in mediating responses to pheromones in goldfish. Although there is some degree of overlap between terminal fields of MOT and

LOT projections, the LOTs primarily project to more lateral telencephalic regions than do the MOTs (Von Bartheld et al., 1984). As suggested by others (Doving and Selset, 1980; Stacey and Kyle, 1983), the LOTs may be more important in mediating feeding responses to food odors.

Central projections of the MOTs terminate in mid-line regions of the ventral telencephalon (Von Bartheld et al., 1984; Levine and Dethier, 1985; Springer, 1983) which have been implicated in the control of a variety of reproductive functions in goldfish and other teleosts (Demski and Hornby, 1982; Kyle et al., 1987). These regions include those that control male reproductive behavior (Kyle et al., 1982; Kyle and Peter, 1982; Koyama et al., 1984), sperm release (Demski and Hornby, 1982) and, more importantly, regions in the preoptic area (POA) that regulate GtH release (Peter et al., 1986). GtH secretion in goldfish is believed to be controlled through the combined actions of gonadotropin-releasing-hormone (GnRH) and dopamine (DA) which stimulate and inhibit GtH release, respectively (Peter et al., 1986). Because the POA contains high concentrations of both GnRH and DA which project to the pituitary (Kah et al., 1986; Kah et al., 1987), a direct MOT projection to the POA may constitute a functional neuroanatomical pathway by which 17,20B-P exerts its action to stimulate GtH release and milt production in goldfish. Circumstantial evidence that olfactory information can reach the POA directly is provided by investigations which demonstrate that electrical stimulation of the OTs cause both sperm release (Demski and Northcutt 1983; Demski and Dulka, 1984) and depletion of neurosecretory material from cell bodies within the POA (Jasinski et al., 1967; Peter and Gorbman, 1968).

In goldfish, selective lesions of the MOTs have been shown to significantly reduce male sexual behavior towards spawning females (Stacey and Kyle, 1983). In addition, lesions placed in two brain regions that receive MOT projections, the *area ventralis telencephali pars supercommisuralis* (Vs) and the *area ventralis telencephali pars ventralis* (Vv), cause significant deficits in male spawning behavior (Kyle and Peter, 1982; Kyle et al., 1982; Koyama et al., 1984). Since male spawning behavior in goldfish is known to be

triggered by the release of a prostaglandin-like female sex pheromone (Sorensen et al., 1988), it is likely that lesions in the Vs-Vv and MOTs disrupt male sexual behavior by blocking this pheromonal input. In contrast, lesions in the LOTs reduce feeding responses to a complex food odor, but have no effect on the performance of male spawning behavior (Stacey and Kyle, 1983). Thus, the MOTs of male goldfish appear to mediate responses to at least two pheromones, each stimulating different endocrine and behavioral responses (Sorensen et al., 1989). It is worth noting that the MOTs also mediate behavioral responses in the female African catfish (*Clarias gariepinus*) to a presumed male sex pheromone which is believed to be steroidal in nature (Resink et al., 1989). The MOTs may therefore play an important reproductive role in a variety of teleosts.

Electrophysiological investigations also provide evidence that the MOTs and LOTs serve different olfactory functions in teleosts. For example, Doving and Selset (1980) demonstrated that electrical stimulation of the isolated MOTs evoke behavioral responses in the cod (*Gadus morhua*) which resemble components of normal spawning behavior. Stimulation of the LOTs, however, evoked increased biting of the gravel substrate as if the fish were searching for food (Doving and Selset, 1980). Furthermore, electrical responses from the MOTs and LOTs in goldfish were recently compared following exposure of the nasal epithelium to a variety of olfactory stimulants (Sorensen, unpublished results). The compounds tested included the 17,20B-P and prostaglandin sex pheromones, the bile salt, taurocholic acid, which acts as a potent olfactory stimulant in fish, and the amino acid, L-serine, which functions as a feeding attractant in goldfish and other teleosts (Caprio, 1984). Only the MOTs responded to the sex pheromones, whereas both tracts responded to the bile salt and amino acid. These findings strongly suggest that the LOTs are primarily involved in mediating responses to food odors while the MOTs play a greater role in social and/or reproductive aspects of communication.

The sperm released following electrical stimulation of the MOTs in goldfish (Demski and Dulka 1984) was originally attributed to activation of the nervus terminalis (NT; Cranial nerve 0) which courses centrally within the MOTs to reach the POA (Demski and Northcutt, 1983). However, it now appears that responses to the 17,20B-P and

prostaglandin pheromones in goldfish are primarily mediated by the olfactory system and not the NT; these substances do not alter the firing pattern of the NT, but cause pronounced increases in the electrical activity of the OBs and the MOTs (Fujita et al., 1989; Sorensen, unpublished results).

In many aspects, the apparent functions of the LOTs and MOTs in goldfish appear analogous to those ascribed to the main olfactory and vomeronasal systems of higher vertebrates (Bertmar, 1981). Although it is debatable whether the vomeronasal organ mediates endocrine and behavioral responses to sex pheromones in mammals, there is considerable evidence to suggest that this system functions in a chemosensory manner (Wysocki, 1979). When the central projections of the main olfactory and vomeronasal systems are compared, it becomes evident that the two systems give rise to totally independent chemosensory pathways. Unlike the main olfactory pathway, which makes connections in the main olfactory bulb, pyriform cortex, thalamus and neocortex, the vomeronasal pathway projects via the accessory olfactory bulb to reach limbic regions in the corticomedial amygdala (Wysocki, 1979). The amygdala in turn makes connections with the ventromedial hypothalamus and POA. Thus, the vomeronasal system projects to brain areas which control pituitary function, as do the MOTs in goldfish. Based on these findings, and growing behavioral information, the vomeronasal system is thought to be involved in a variety of physiological and social functions related to reproduction (Wysocki, 1979; Meredith, 1983). However, it is still unclear whether the vomeronasal system plays a more important role than the main olfactory system in mediating responses to sex pheromones in mammals. Taken together, the available information suggests that the LOTs in goldfish serve a function analogous to the main olfactory system in higher vertebrates. In contrast, the MOTs and their central projections comprise a pathway that is remarkably similar to the vomeronasal system. In this regard, the Vs-Vv regions of the ventral telencephalon of teleosts, which receive MOT projections, are considered to be homologous to the basal amygdala in higher vertebrates (Northcutt, 1981). Therefore, MOT projections in goldfish may exist either as evolutionarily conserved pathways which are retained in higher vertebrates as the vomeronasal system, or as new pathways which

have evolved independently to serve the same functions. Whether the MOTs and the vomeronasal systems serve the same reproductive functions remains to be determined. In this regard, the 17,20B-P pheromone system in goldfish appears to be an excellent comparative model for the study of vertebrate sex pheromones.

In summary, the MOTs play an indispensable role in mediating GtH and milt responses to water-borne 17,20B-P in male goldfish. This olfactory subdivision and its connections with the POA may constitute the neuroanatomical pathway by which 17,20B-P stimulates GtH release to ultimately increase milt production by the time females ovulate (Dulka et al., 1987; Stacey et al., 1989). A reproductive role of the MOTs does not appear to be restricted to goldfish since considerable evidence suggests that this olfactory subdivision mediates reproductive responses to sex pheromones in other teleosts as well (Kyle et al., 1987). In addition, the projections of the MOTs may serve a function that is analogous to the vomeronasal system in higher vertebrates.

Figure 3.1 Effect of various olfactory tract (OT) lesions on plasma GtH (**A**) and milt volume (**B**) following exposure to 17,20B-P two days after surgery. INTACT: unoperated fish, both OTs intact; SHAM: sham operated, both OTs intact; LOTX: both lateral olfactory tracts (LOTs) cut, medial olfactory tracts (MOTs) intact; MOTX: MOTs cut, LOTs intact; OTX: both OTs severed completely. All fish were blood sampled 1 h after exposure to EtOH or 17,20B-P. Milt was stripped 12 h later. n=10-12 in all groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to the EtOH exposed controls for each surgical treatment (ANOVA-Least Squares Means analysis).

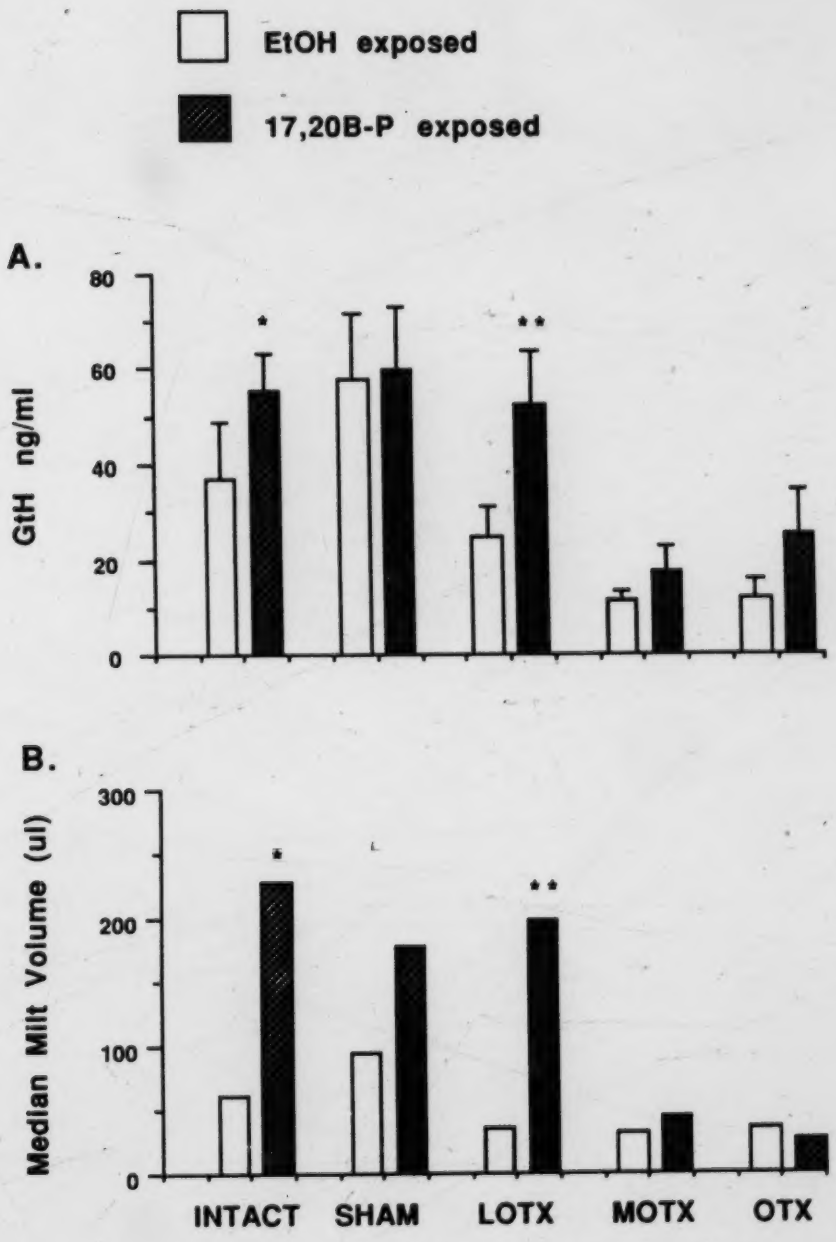
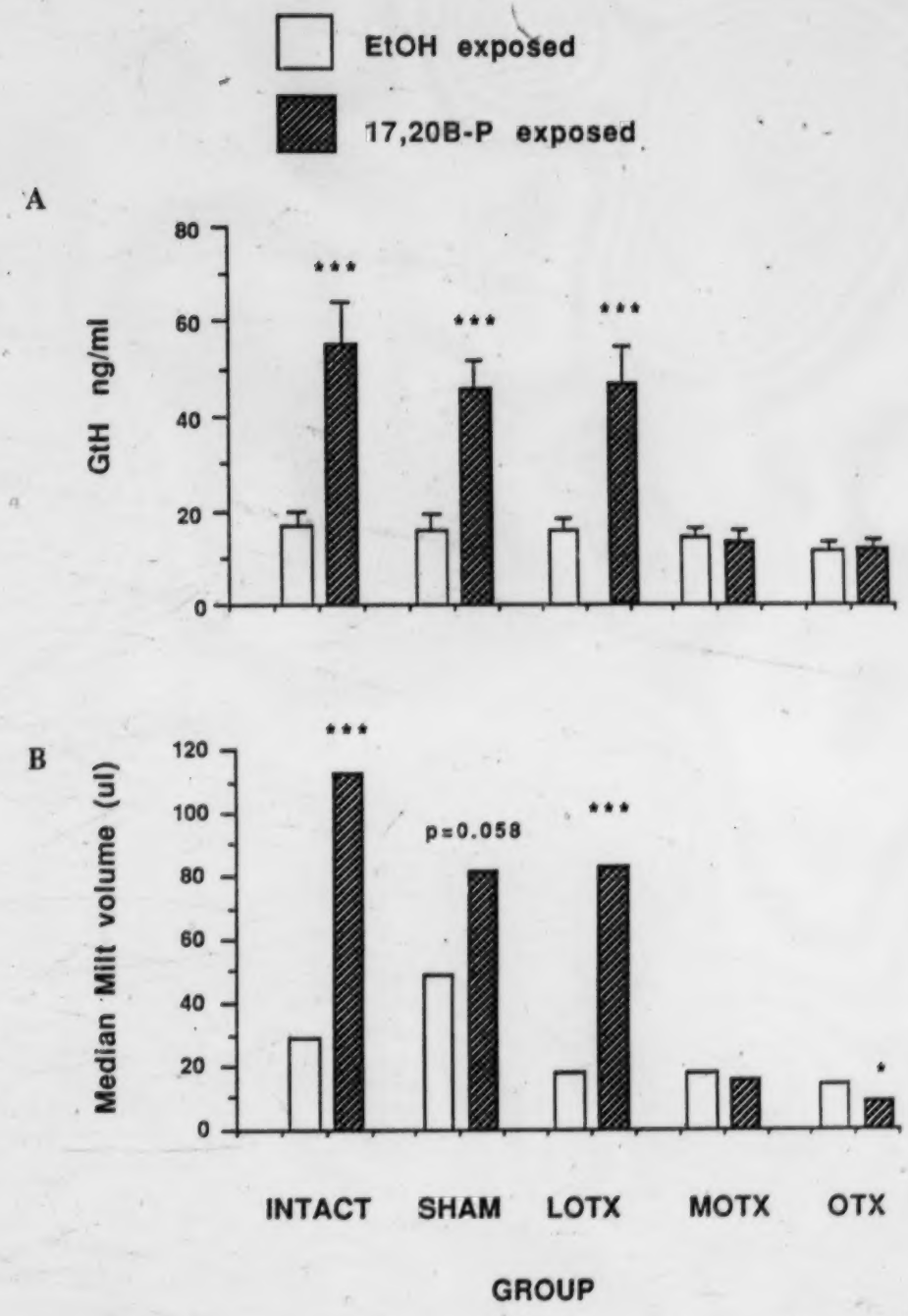

EtOH exposed
17,20B-P exposed
A.
GtH ng/ml
80
60
40
20
0
*
**
B.
Median Milt Volume (ul)
300
200
100
0
*
**
INTACT
SHAM
LOTX
MOTX
OTX

Figure 3.2 Effect of various olfactory tract lesions on plasma GtH (**A**) and milt volume (**B**) in male goldfish following exposure to either EtOH or 17,20B-P one week after surgery. See figure 5.1 for explanation.


EtOH exposed
17,20B-P exposed
A
GtH ng/ml
80
60
40
20
0
***
***
***
B
Median Milt volume (ul)
120
100
80
60
40
20
0
***
p=0.058
***
*
INTACT
SHAM
LOTX
MOTX
OTX
GROUP

3.5 LITERATURE CITED

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4. CHARACTERIZATION OF MALE ENDOCRINE RESPONSES TO THE 17α,20B-P FEMALE SEX PHEROMONE IN GOLDFISH: DOSE-RESPONSE RELATIONSHIPS [1]

4.1 INTRODUCTION

We have recently demonstrated that the gonadal steroid, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P) acts as a potent female sex pheromone in goldfish, *Carassius auratus* (Stacey and Sorensen, 1986; Dulka et al., 1987; Stacey et al., 1989). This steroid, which functions endogenously to promote oocyte final maturation (Nagahama et al., 1983), increases in the blood of females approximately 10-12 h before ovulation (Kobayashi et al., 1987; Stacey et al., 1989), and is simultaneously released to the water by preovulatory females (Dulka et al., 1987; Van Der Kraak et al., 1989; Stacey et al., 1989) where it functions as a potent olfactory stimulant (Sorensen et al., 1987; Sorensen et al., 1989a). Males exposed to water-borne 17,20B-P increase blood gonadotropin (GtH) within 15 min and milt (sperm and seminal fluid) production within 4-6 h (Dulka et al., 1987). In addition, the GtH and milt responses normally induced by 17,20B-P exposure are abolished by cutting the medial olfactory tracts, which previously have been implicated in the control of sexual responses to pheromones in goldfish (Stacey and Kyle, 1983; Demski and Northcutt 1983) and other teleosts (Doving and Selset, 1980; Resink, 1988). Based on these findings, we have proposed that water-borne 17,20B-P functions as a pheromone which synchronizes milt production with ovulation in goldfish (Stacey and Sorensen, 1986; Dulka et al., 1987; Stacey et al., 1989).

1. A version of this chapter has been submitted for publication. Sorensen, P.W., N.E. Stacey, T.J. Hara, and J.G. Dulka. 1989. J. Comp. Physiol.

Electrical recordings from the olfactory epithelium (electro-olfactogram; EOG) of goldfish have established 17,20B-P as the most potent olfactory stimulant described in fish with a detection threshold of approximately 10^{-12} M (Sorensen et al., 1987; Sorensen et al., 1989a). The present investigation examined the detection threshold at which males respond to water-borne 17,20B-P by monitoring GtH responses to various concentrations of the pheromone under two testing conditions: (1) by adding doses of 17,20B-P directly to aquaria containing fish, and (2) by first pre-diluting the steroid doses in test aquaria before adding fish. Growth hormone (GH) levels also were measured for two reasons. First, although GH levels in male goldfish have not been examined during reproduction, I suspected that GH may increase in the blood of males exposed to 17,20B-P because GH increases in parallel with the preovulatory GtH surge in females (Marchant, 1983). Second, results from *in vivo* and *in vitro* studies indicate that gonadotropin-hormone releasing-hormone (GnRH) stimulates the release of both GtH and GH in male and female goldfish (Marchant, 1988). Based on these findings, I examined whether GtH and GH increase simultaneously in the blood of males exposed to 17,20B-P in order to better understand the central regulation of the GtH response to the pheromone.

4.2 MATERIALS AND METHODS

Animals

Goldfish of the common or comet variety (20-30 g) were purchased from Ozark Fisheries Co., Stoutland, Missouri. Stocks of males were maintained in 1000 L flow-through aquaria at 15-20° C under a 16 h light: 8 h dark photoperiod. Fish were fed Nutrafin flaked food or Ewos trout pellets *ad libitum* once a day. Only spermiated males having well developed pectoral tubercles (pearl organs) and expressible milt (sperm and seminal fluid) were used in the experiments.

Experiment 1.

This experiment, conducted in May, tested the effects of various doses of water-borne 17,20B-P on GtH release in male goldfish. Following established procedures (Dulka et al., 1987), different amounts of 17,20B-P were added directly to aquaria containing fish. One day before the experiment, groups of males were placed (3 fish per aquarium) in 65 L flow-through test aquaria at 20° C. All aquaria contained gravel substrate and a vigorously bubbling aeration stone. At the beginning of scotophase (01:00 h) on the following day, the water flow to all aquaria was shut off. One group of fish was exposed to 100 µl of ethanol (control) while the remaining males were exposed to either 0.005, 0.05, 0.5, 5.0 or 50.0 µg 17,20B-P in 100 µl of ethanol. Although the resulting 17,20B-P water concentrations were not measured, these doses should have created concentrations of 17,20B-P ranging from 10^{-13} to 10^{-9} M in log molar steps. To promote rapid dilution, the ethanol and 17,20B-P solutions were injected directly into the column of bubbles which originated from the aeration stone in each aquarium. Sixty min later, all fish were anesthetized in a 0.05% solution of 2-phenoxyethanol (Syndel Laboratories, Vancouver, British Columbia) and blood sampled through the caudal vasculature for determination of plasma GtH concentrations. The experiment was completed within a 24 h period.

Experiment 2.

This experiment, which was conducted in July on the same stock of fish used in experiment 1, tested the effects of various doses of 17,20B-P on both GtH and GH release in male goldfish. Procedures were modified to determine whether the males in experiment 1 might have responded to "concentrated" 17,20B-P odor plumes prior to complete dilution of the steroid in the water. Rather than adding odors to aquaria containing fish, the ethanol and steroid solutions were first injected into empty aquaria, and, following a 5.0 min period to allow complete dilution, groups of 3 males were added. Thirty min later, all fish were anesthetized and blood sampled for determination of plasma GtH and GH concentrations. Because twice as many aquaria were required as in experiment 1, this experiment was conducted in three separate runs over a one week period. Each run

contained one third of the sample size for each treatment group. All aquaria were drained and rinsed thoroughly with flowing water for at least 24 h before the next successive run was conducted.

Hormone Assays

Blood samples were allowed to clot for several hours at 4° C and then centrifuged to obtain serum which was stored frozen (-25° C) until assayed. Serum GtH concentrations were determined by radioimmunoassay (RIA) as described previously (Peter et al., 1984). Serum GH levels were determined by RIA as described by Marchant et al. (1987).

Statistical Analysis

GtH and GH values were log transformed and analysed by ANOVA. Individual comparisons between ethanol and 17,20B-P exposed groups were performed using Newman-Keuls follow-up tests.

4.3 RESULTS

Experiment 1.

When 17,20B-P was added to aquaria containing fish, only those males exposed to final water concentrations of 10^{-12} M or greater had significantly ($p<0.05$) higher GtH levels than ethanol-exposed controls (Fig 4.1A). Once the threshold concentration (10^{-12} M) of 17,20B-P was reached, there was no indication of further GtH increases to higher doses of the pheromone (Fig. 4.1A).

Experiment 2.

When males were added to aquaria containing pre-diluted 17,20B-P, only those males exposed to water concentrations of 10^{-11} M or greater had significantly ($p<0.05$)

higher GtH levels than ethanol-exposed controls (Fig. 4.1B). Unlike the first experiment, the 0.05 µg/tank dose of 17,20B-P (10^{-12} M) had no effect on GtH release. However, as in the first experiment, the magnitude of the GtH responses were similar in all groups exposed to supra-threshold concentrations of 17,20B-P (10^{-11}-10^{-9} M). Serum GH levels were not affected by 17,20B-P exposure (Fig 4.1B).

4.4 DISCUSSION

The present study provides direct functional evidence that male goldfish are extremely sensitive to low concentrations of water-borne 17,20B-P. The finding that GH dose not change following exposure to 17,20B-P suggests that the pheromone functions to specifically stimulate GtH release in males and indicates that the central regulation of the GtH response to 17,20B-P differs from that controlling the preovulatory GtH surge in females. In addition, the olfactory sensitivity to 17,20B-P as indicated by increased GtH release is remarkably similar to that indicated by EOG recordings (Sorensen et al. 1987; Sorensen et al., 1989a). Of a total of 24 steroids tested using EOG techniques, 17,20B-P was found to be the most potent at evoking changes in the electrical activity of the olfactory epithelium (Sorensen et al., 1989a). Indeed, with a detection threshold of approximately 10^{-12} M, 17,20B-P is the most potent olfactory stimulant reported in fish (Sorensen et al., 1987; Sorensen et al., 1989a). 17α-hydroxyprogesterone (17αP), the precursor of 17,20B-P, and 17α,20B,21-triol-4-pregnen-3-one (17α,20B,21-P), a metabolite of 17,20B-P reported in some species of fish (Thomas et al., 1987), were also found to be highly stimulatory when tested using EOG (Sorensen et al., 1989a). The responses to these compounds appear specific since other related steroids which either lack a hydroxyl group in the 17 and/or 20 carbon positions, or possess them in different configurations (either α or B), are much less potent as olfactory stimulants when tested in both EOG and endocrine bioassay systems (Sorensen et al., 1989a). Finally, cross-adaption experiments demonstrate that responses to 17,20B-P-like compounds are mediated by a single class of

olfactory receptors which exhibit the highest affinity for 17,20B-P (Sorensen et al., 1987; Sorensen et al., 1989a). Taken together, these and the present findings suggest that 17,20B-P (and perhaps 17α,20B,21-P) functions as a highly specific pheromone which signifies impending ovulation in females and causes rapid endocrine changes in males which lead to increased milt production by the time of ovulation (Dulka et al., 1987).

In the present study, the threshold concentration required for GtH release in the first Experiment (10^{-12} M) was lower than that required in the second Experiment (10^{-11} M). Since 17,20B-P was added directly to aquaria containing fish in Experiment 1, whereas it was pre-diluted in Experiment 2, the apparent discrepancy between threshold concentrations is believed to have resulted from fish in Experiment 1 encountering supra-threshold 17,20B-P "plumes" prior to complete dilution of the steroid. As I have observed that similar injections of water soluble dyes are mixed to homogeneity within 30 s, the GtH response induced by 10^{-12} M 17,20B-P in Experiment 1 likely occurred after only a brief exposure to the supra-threshold "plumes", since the same 17,20B-P dose failed to elicit a response after a 5.0 min pre-dilution period in Experiment 2. The possibility remains, however, that stress associated with moving fish to the pre-diluted aquaria may have somehow increased response threshold in experiment 2.

Although GtH responses to water-borne 17,20B-P occur at concentrations comparable to those required to activate olfactory receptors, the responses we observe following direct addition of the pheromone to the water may not necessarily reflect those which occur under natural conditions. For example, although we have demonstrated that preovulatory female goldfish release considerable amounts of 17,20B-P to the water (Dulka et al., 1987; Van Der Kraak et al., 1989; Stacey et al., 1989), it is not known whether the pheromone is released in a continuous or pulsatile manner. Recent calculations indicate that if the pheromone is released at a constant rate, the amount present in the water would be sufficient to produce only a small and short-lived odor plume which would be detectable only for a few seconds and, especially at moderate swimming speeds, only within a few centimeters of the female (Sorensen and Stacey, 1989). However, by actively chasing females, males may encounter many pheromone pulses and extend their exposure time to

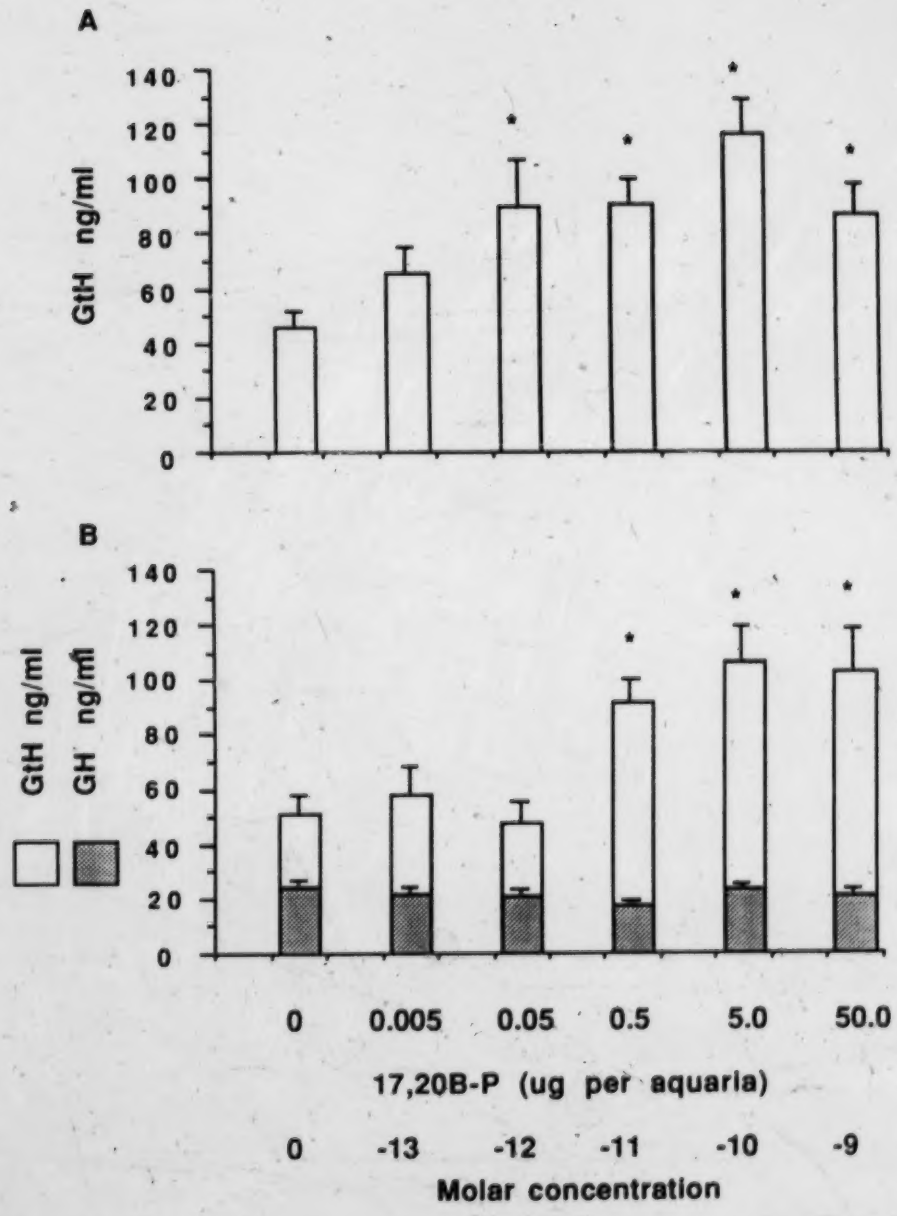
the pheromone. Even so, the method of chronic 17,20B-P exposure normally employed in this and my other investigations is unlikely to realistically simulate the pattern of stimulation males receive from preovulatory females. Unfortunately, it is not known whether a brief (e.g. 1 min) pheromone pulse is as effective as prolonged (e.g. several hours) exposure, or whether repetitive stimulations might have additive effects on GtH release and milt production.

In both experiments, the difference between non-stimulatory and stimulatory (threshold) concentrations of 17,20B-P was one log molar unit, indicating that if males have graded GtH responses to the pheromone they occur over a relatively small concentration range (i.e. 10^{-12} to 10^{-11} M). In addition, a continued dose-dependent pattern of GtH release to increasing, supra-threshold concentrations of 17,20B-P was not observed, since higher concentrations of the pheromone were no more effective at elevating GtH than were threshold concentrations. This finding may not be surprising if one considers that, because the concentration of 17,20B-P encountered by a male will be influenced by the stage of oocyte maturation (Stacey et al., 1989), the mode of pheromone release (continuous or pulsatile), the swimming speed of the female, and the distance from the female (Sorensen and Stacey, 1989), selection pressures may have enabled males to simply increase GtH in response to stimulatory concentrations of the 17,20B-P, but not to have graded responses to supra-threshold concentrations of the pheromone.

In contrast to GtH release, EOG recordings in goldfish clearly demonstrate that olfactory receptors show marked dose-dependent responses to brief pulses of increasing, supra-threshold concentrations (10^{-12} to 10^{-7} M) of water-borne 17,20B-P (Sorensen et al., 1987; Sorensen et al., 1989). It is possible that the activity of the olfactory receptors enables males to detect a 17,20B-P concentration gradient and thus keep in close physical contact with 17,20B-P-releasing females. In this regard, water-borne 17,20B-P has been shown to have subtle, but significant, effects on male sexual arousal (Sorensen et al., 1989b).

In summary, the threshold water concentration of 17,20B-P necessary to induce a GtH response (approximately 10^{-11} M) is similar to that determined electrophysiologically from the olfactory epithelium (Sorensen et al., 1987; Sorensen et al., 1989a). Above threshold 17,20B-P concentrations, males do not show dose-dependent GtH responses to the pheromone. Absence of a dose-response in GtH release is similar to the situation with 17,20B-P-induced milt responses (Stacey and Sorensen, 1986; Sorensen et al., 1989a), but clearly different from that exhibited by the olfactory epithelium as demonstrated by EOG recordings (Sorensen et al., 1987; Sorensen et al., 1989a). The reason why males show maximal GtH and milt responses to threshold concentrations of the pheromone, but exhibit dose-dependent EOG responses to increasing concentrations (i.e. 10^{-12} to 10^{-8} M) of water-borne 17,20B-P remains to be elucidated, although these differences may be related to behavioral effects of 17,20B-P which are poorly understood. The results suggest that male goldfish are capable of responding to brief pulses of short-lived, supra-threshold concentrations of 17,20B-P, although it is not known whether females release 17,20B-P to the water in a continuous or pulsatile manner. Taken together, the extreme olfactory sensitivity of male goldfish to water-borne 17,20B-P and their accompanying GtH and milt responses further establishes this pheromone system as a unique comparative model to study pheromone function in vertebrates in general.

Figure 4.1. **A**. Influence of a 1 h exposure to various doses of water-borne 17,20B-P on GtH secretion in male goldfish. 17,20B-P was added directly to aquaria containing males. **B**. Influence of a 30 min exposure to various doses of water-borne 17,20B-P on serum GtH (clear bars) and GH (shaded bars) in male goldfish. The different doses of 17,20B-P were first pre-diluted in test aquaria before fish were added. In both **A** and **B**, control fish (zero 17,20B-P) were exposed to 100 ul ethanol alone. The amount (µg/100 ul ethanol) of 17,20B-P added for each group and the resulting calculated final water concentrations are indicated below the figure. The same blood samples were assayed for GtH and GH. Statistical differences between experimental and control groups for GtH are indicated above the bars (*$p<0.05$, Newman-Keuls procedure). GH levels in **B** were similar in all groups ($p>0.05$, ANOVA).


A
GtH ng/ml
140
120
100
80
60
40
20
0
B
GtH ng/ml
GH ng/ml
140
120
100
80
60
40
20
0
0
0.005
0.05
0.5
5.0
50.0
17,20B-P (ug per aquaria)
0
-13
-12
-11
-10
-9
Molar concentration

4.5 LITERATURE CITED

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5. CHARACTERIZATION OF MALE ENDOCRINE RESPONSES TO THE 17,20B-P FEMALE SEX PHEROMONE IN GOLDFISH: TIME-COURSE OF PHEROMONALLY-INDUCED ENDOCRINE RESPONSES IN MALES

5.1 INTRODUCTION

The gonadal steroid, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), which functions endogeneously to promote final oocyte maturation in females (Nagahama et al., 1983; Goetz, 1983), is also released to the water where it functions as a potent female sex pheromone in goldfish (Stacey and Sorensen, 1986; Dulka et al., 1987a; Sorensen et al., 1987; Stacey et al., 1989). When added directly to aquarium water, 17,20B-P and 17,20B-P-like compounds, but not a variety of other steroids, cause a dramatic increase in milt (sperm and seminal fluid) production in males (Stacey and Sorensen, 1986). Pheromonal 17,20B-P exerts its action through the olfactory system since bilateral olfactory tract section blocks the milt response to water-borne 17,20B-P (Stacey and Sorensen, 1986; Chapter 3). In addition, the olfactory epithelium of males is extremely sensitive to water-borne 17,20B-P (detection threshold of approximately 10^{-13}-10^{-12} M; Sorensen et al., 1987).

Recently, Dulka et al., (1987a) demonstrated that the milt response to 17,20B-P is mediated by acute increases in blood gonadotropin (GtH) in males. Hypophysectomy blocks the milt response to water-borne 17,20B-P and males exposed to the pheromone exhibit increases in GtH within 15 min and subsequent increases in milt volume within 6 h (Dulka et al., 1987a). Elevated levels of plasma GtH are believed to act through testicular mechanisms to promote increased milt production. In this regard, the 17,20B-P-induced GtH response in male goldfish is accompanied by an increases in blood 17,20B-P (Dulka et al., 1987a). This observation is consistent with the proposal that testicular 17,20B-P mediates the action of GtH on milt production in goldfish and some other teleosts (Ueda et

al., 1985). Since preovulatory females begin to release 17,20B-P 8-10 h prior to ovulation (Stacey et al., 1989), the latency of male GtH and milt responses to the pheromone allows milt production to be synchronized with ovulation (Dulka et al., 1987a).

The male endocrine responses to water-borne 17,20B-P appear specific for GtH (and milt production) since growth hormone (GH) levels do not change in males exposed to the pheromone (Dulka et al., 1987b; Chapter 4). However, the effects of water-borne 17,20B-P on GtH and GH release have been examined over a standardized 1 h exposure period (Chapter 4), rather than over time that the pheromone would normally act. In addition, although we have clearly demonstrated a direct relationship between 17,20B-P exposure, increased GtH release, and the occurrence of increased milt production, we have yet to determine the precise time-course and specificity of endocrine changes in males exposed to the pheromone. In this study, temporal changes in circulating levels of GtH, GH, testosterone (T), and 17,20B-P of presumed testicular origin were measured in males during several hours of continuous exposure to water-borne 17,20B-P. In addition, the time-course of the milt response to water-borne 17,20B-P was re-examined.

5.2 MATERIALS AND METHODS

Animals:

Goldfish of the common or comet variety (20-30 g) were purchased from Ozark Fisheries Co. Stoutland, Missouri. Sexually mature males having well developed pectoral tubercles (pearl organs) and expressible milt were maintained in 1000 L flow-through stock aquaria at 15-20^{o} C under a 16L:8D photoperiod (lights on at 08:00 h). Fish were fed Ewos trout pellets and Nutrafin flaked food *ad libitum* at least once a day.

Experiment 1

This experiment examined temporal changes in serum concentrations of GtH, GH, T, and 17,20B-P in male goldfish following exposure to water-borne 17,20B-P. The latency

of the 17,20B-P-induced milt response was also examined in the same animals. On day 1, groups of males were transferred to 65 L flow-through test aquaria (3 fish/aquarium) at 20° C. Groups of 4 aquaria (12 fish) were then assigned to one of 8 treatment groups depending on how long they would be exposed to ethanol or 17,20B-P. The exposures began at 09:00 h on day 2. Briefly, the water flow to the appropriate aquaria was shut off and either 100 µl of ethanol or 10 µg 17,20B-P in 100 µl ethanol was added to the water. To promote rapid dilution, the ethanol and 17,20B-P solutions were injected directly into the column of bubbles which originated from aeration stones in each aquarium. Although the resulting 17,20B-P water concentrations were not measured, the amount added to each aquarium should have created a final water concentration of approximately $5X10^{-10}$ M. The different groups of control fish were anesthetized (0.05% solution of 2-phenoxyethanol, Syndel) and blood sampled through the caudal vasculature following either 15 min, 2 h, or 8 h of exposure to ethanol alone. The different groups of experimental males were blood sampled following either 15 min, 45 min, 2 h, 4 h, or 8 h of exposure to 17,20B-P. Immediately after blood sampling, the amount of milt which could be stripped from each fish was collected in pre-weighed hematocrit tubes which were then fitted with weighed caps as described previously (Stacey and Sorensen, 1986). The filled tubes were then weighed and the weight of milt calculated. Assuming a milt density of 1.0, the milt values are expressed as volumes (µl) rather than weights.

Experiment 2

This experiment was conducted to verify the 17,20B-P-induced endocrine and milt changes observed in Experiment 1 and specifically examined whether blood levels of 17,20B-P also change in males exposed to water-borne 17,20B-P. The procedures used to distribute fish in test aquaria (day 1) were exactly the same as those described for Experiment 1 (see above). Groups of fish were then assigned to one of 3 control or one of 7 experimental treatment groups depending on how long they would be exposed to ethanol or 17,20B-P. Between 09:00-11:00 h on day 2, the amount of milt which could be stripped from each fish was collected to establish pre-exposure levels of milt in each treatment

group. The exposures to ethanol and 17,20B-P began at 09:00 h on day 3, as described above. Control fish were blood sampled after either 30 min, 2 h, or 8 h exposure to ethanol alone. Experimental males were blood sampled following either 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, or 12 h of exposure to 17,20B-P. Immediately after blood sampling, the amount of milt which could be stripped from each fish was collected (see above) to determine the latency of the milt response to water-borne 17,20B-P.

Experiment 3

This experiment examined only the time course of the milt response to water-borne 17,20B-P. On the morning (09:00 h) of day 1, groups of males were transferred to test aquaria (3 fish/ aquarium) as described above (Experiment 1). The fish were then randomly assigned to one of 4 treatment groups. Between 19:00 h-23:00 h on the same day, the amount of milt which could be stripped from each fish was collected to establish pre-exposure levels of milt volume in each group. Beginning at 09:00 h on day 2, the water flow to each aquarium was shut off. One half of the males in each group was exposed to 100 µl of ethanol while the other half was exposed to 10 µg 17,20B-P in 100 µl ethanol. After either 2 h, 4 h, 6 h, or 8 h of exposure, the paired ethanol and 17,20B-P-exposed groups were again stripped of milt to determine the latency of the milt response to water-borne 17,20B-P.

Experiment 4

This experiment was designed to more accurately determine the latency of the milt response to water-borne 17,20B-P. The experiment was conducted exactly as Experiment 3 except that the control and experimental groups were exposed to ethanol or 17,20B-P for either 1 h, 2 h, 4 h, or 6 h, respectively.

Hormone Assays

Serum GtH and GH concentrations were determined by radioimmunoassay (RIA) as described previously (Peter et al., 1984; Marchant et al., 1988). For steroid measurement, serum samples were extracted two times with 2 volumes of diethyl ether and the amount of T and 17,20B-P in the extract determined by RIA as described by Van Der Kraak et al. (1984).

Statistical Analysis

All data were normalized using a logarithmic transformation. In experiment 1 and 2, GtH, GH, T, and 17,20B-P data were analysed by 2-way analysis of variance (ANOVA) and Least Squares Means follow-up tests using a SAS computer package (SAS Institute Inc. Cary, NC). In Experiments 3 and 4, the pre-exposure milt data were analysed by one-way ANOVA since the animals had received no treatment. The milt data obtained following exposure to ethanol or 17,20B-P were analysed by 2-way ANOVA and Least Squares Means analysis.

5.3 RESULTS

Experiment 1

Serum GtH levels in males exposed to ethanol (Control) for 15 min, 2 h, and 4 h remained low and were not significantly ($p>0.05$) different (Fig. 5.1A). In contrast, exposure to 17,20B-P significantly ($p<0.05$) elevated GtH in males at each sample time (Fig. 5.1A). The magnitude of the GtH response was similar in all 17,20B-P-exposed groups (Fig. 5.1A).

GH levels in control males were similar ($p>0.05$) at all sample times (Fig. 5.1B). Serum GH levels in males exposed to 17,20B-P were similar ($p>0.05$) to those of the ethanol-exposed groups after 15 min, 45 min, 2 h, and 4 h of exposure. In contrast, GH levels in males exposed to 17,20B-P for 8 h were significantly ($p<0.05$) higher than those

of their respective control group (Fig. 5.1B).

Serum T levels were similar in all ethanol-exposed groups (Fig. 5.1C). Males exposed to 17,20B-P for 15 min and 45 min had similar ($p>0.05$) levels of T compared to control animals exposed to ethanol for 15 min and 2 h. In contrast, males exposed to 17,20B-P for either 2 h, 4 h, or 8 h had equivalent T levels which were significantly ($p<0.01$) higher than those of all other groups (Fig. 5.1C).

The amount of plasma which remained after determination of T concentrations in each blood sample generally was insufficient to allow subsequent measurement of plasma 17,20B-P. Thus, although the remaining plasma samples were extracted and assayed for 17,20B-P, plasma levels of 17,20B-P in most groups were lower than the detection limit of the RIA (150.0 pg/ml using a 200 ul plasma sample; data not shown).

Milt volumes in control groups remained low and were not significantly different ($p>0.05$) following exposure to ethanol for 15 min, 2 h, or 8 h (Fig. 5.1D). The milt volumes of males exposed to 17,20B-P for 15 min and 45 min were similar to those of males exposed to ethanol for 15 min and 2 h. The milt volume of males exposed to 17,20B-P was significantly ($p<0.05$) less than that of ethanol-exposed controls at 2 h. However, males exposed to 17,20B-P for either 4 h or 8 h had significantly ($p<0.05$) higher milt volumes than males exposed to ethanol for 8 h (Fig. 5.1D).

Experiment 2

Serum GtH levels in males exposed to ethanol (Control) for 30 min, 2 h and 8 h remained low and were not significantly ($p>0.05$) different (Fig. 5.2A). In contrast, exposure to water-borne 17,20B-P significantly ($p<0.05$) elevated GtH in males at each sample time (Fig. 5.2A).

GH levels were similar in males exposed to ethanol for 2 h and 8 h, but significantly ($p<0.05$) lower in males exposed to ethanol for 30 min (Fig. 5.2B). Serum GH levels in males exposed to 17,20B-P were similar, and not significantly different than groups exposed to ethanol at each sample time (Fig 5.2B).

Blood 17,20B-P levels were consistently low and similar in all ethanol-exposed

groups (Fig. 5.2C). In contrast, exposure of males to water-borne 17,20B-P significantly ($p<0.05$) elevated blood levels of 17,20B-P at each sample time except for those males exposed to the pheromone for 8 h (Fig. 5.2C).

Milt volumes in the control groups remained low and were not significantly ($p>0.05$) different (Fig. 5.2D). Only those males exposed to water-borne 17,20B-P for 8 h and 12 h had significantly ($p<0.05$) higher milt volumes than those of the ethanol-exposed controls (Fig. 5.2D).

Experiment 3

Pre-exposure milt volumes did not differ among groups (Fig. 5.3A). Post-exposure milt volumes in males exposed to ethanol alone remained low and were not significantly different, regardless of exposure time (Fig. 5.3B). Only males exposed to 17,20B-P for 6 h and 8 h had significantly ($p<0.05$) higher milt volumes than their respective control groups (Fig. 5.3B).

Experiment 4

Pre-exposure milt volumes were similar ($p>0.05$) in all groups (Fig. 5.4A). Post-exposure milt volumes were similar in all groups exposed to ethanol alone, regardless of the time of exposure (Fig. 5.4B). The milt volume of males exposed to 17,20B-P for 1 h was not significantly different from that of ethanol-exposed controls. In contrast, males exposed to 17,20B-P for either 2 h, 4 h, or 8 h had significantly ($p<0.05$) higher milt volumes than their respective ethanol-exposed groups (Fig. 5.4B).

5.4 DISCUSSION

Gonadotropin

The results of the present study confirm our previous findings (Stacey and Sorensen, 1986; Dulka et al., 1987) that water-borne 17,20B-P rapidly stimulates GtH release and milt production in male goldfish. The GtH response to 17,20B-P appears to occur immediately after the pheromone is detected; exposure to 17,20B-P for as little as 15 min is sufficient to cause a two-fold increase in blood GtH. Surprisingly, blood GtH levels remain elevated in 17,20B-P-exposed males 8-12 h after initial exposure. In addition, the magnitude of GtH responses to water-borne 17,20B-P remains constant regardless of the duration of exposure.

Both the speed and persistence of the GtH response to water-borne 17,20B-P raise some important questions as to how the pheromone triggers GtH release in goldfish. Under our present testing conditions, males would be expected to encounter stimulatory concentrations of 17,20B-P within 30-40 s of the steroid being injected to the water, because similar injections of water soluble dyes are mixed to homogeneity within 30 s (personal observations). In addition, doses (0.05 µg/aquarium) of 17,20B-P, which are ineffective at evoking male GtH responses when first pre-diluted in the water (approximate concentration, 10^{-12} M), are effective at eliciting GtH responses when injected directly to aquarium water containing males (Chapter 4). The responses observed in the latter situation are believed to be due to males encountering supra-threshold 17,20B-P odor "plumes" before the steroid is diluted below detectablity (Chapter 4). Taken together, the results indicate that males require only a brief exposure to water-borne 17,20B-P to initiate increased GtH release.

The mechanism whereby GtH levels remain elevated following extended (8-12 h) exposure to 17,20B-P is not understood. It is not known whether the amount (10 µg/aquarium) of 17,20B-P added to the water remains stimulatory, or whether it is degraded or absorbed by organisms in the aquarium. Neither is it known whether males maintain their ability to detect and respond to water-borne 17,20B-P during extended

exposure, or whether male olfactory receptors adapt to stimulatory concentrations of water-borne 17,20B-P. Although EOG responses to 17,20B-P exhibit both phasic and tonic components, Sorensen et al. (1987) observed little or no reduction in the tonic component of the EOG response following lengthy exposure to 17,20B-P, whereas the phasic component decreased in magnitude. This may suggest that, although 17,20B-P receptors modify their electrical activity to 17,20B-P, the central input from these receptors may be sufficient to maintain increased GtH release. Although males continue to release high amounts of GtH during continuous exposure to 17,20B-P, it is not known if males are capable of maintaining increased GtH release after only a brief exposure to the pheromone. However, in Chapter 2, males exposed to 17,20B-P for 15 min and 2 h had significantly elevated levels of milt which were similar to each other 7-8 h later (Fig. 2.3), suggesting that only a brief exposure to 17,20B-P is sufficient to increase milt volume.

Conversely, if one assumes that goldfish lose their ability to detect 17,20B-P during extended exposure, a regulatory mechanism, other than that associated with the olfactory receptors, would be required to account for increased GtH over 8-12 h of chronic exposure. This mechanism may involve additional neural processing of pheromonal information in regions of the hypothalamus that control GtH release (Peter et al., 1986). Perhaps only brief pheromonal input to the hypothalamus activates a "neuroendocrine program" for increased GtH release which runs independently of further pheromonal input.

In this regard, the GtH response to 17,20B-P in male goldfish may be analogous to neuroendocrine reflexes in some mammalian species. For example, in rabbits, peripheral stimulation (penile thrusting) of the vagina appears to be sufficient to cause reflex ovulation within one hour of copulation (see review: Allen and Adler, 1985). The sensory input of vaginal stimulation converges through the spinal cord to reach areas of the medial basal hypothalamus that control the ovulatory GtH surge. Vaginal stimulation induces increased multiunit activity in the arcuate, premammillary and posterolateral hypothalamic nuclei, whereas the same stimulation causes reduced activity in the medial POA and ventromedial hypothalamus (see review: Allen and Adler, 1985). Electrical brain stimulation and lesion studies have verified the involvement of these areas in controlling the ovulatory GtH surge

in rabbits and other mammalian species (see review: Allen and Adler, 1985). Thus, in this example, the peripheral nervous system conveys somatosensory stimuli to the central nervous system which transduces these stimuli into immediate and long-term hormonal changes by altering the functional activity of the hypothalamic-pituitary-gonadal axis. Since water-borne 17,20B-P appears to trigger similar endocrine changes in male goldfish, it is proposed that increases in GtH occur as a result of a reflex response to the pheromone.

The temporal pattern of GtH release in the present study is similar to that observed under simulated natural conditions. Males exposed to preovulatory 17,20B-P-releasing females show increased concentrations of blood GtH when levels of 17,20B-P first begin to increase in the water (8-10 h prior to ovulation, Stacey et al., 1989). GtH levels in these males remain elevated throughout the preovulatory period and appear to play an important role in stimulating milt production prior to spawning (Stacey et al., 1989, see below).

Growth Hormone

Unlike blood GtH, which increases rapidly and consistently in response to water-borne 17,20B-P, male GH levels do not appear to be directly affected by the pheromone. However, water-borne 17,20B-P may exert indirect effects on GH release. For example, males in Experiment 1 exhibited increased levels of GH, but only after 8 h of continuous exposure to the pheromone (Fig 5.1C). Similar changes in GH concentrations, however, were not observed in Experiment 2 (Fig. 5.2B). Since goldfish do not exhibit a daily rhythm of GH release (T.A. Marchant and R.E. Peter, personal communication), one explanation for this apparent effect on GH in Experiment 1 is that 17,20B-P-induced GtH responses cause increased production of gonadal steroids which, in turn, cause increased GH release through a positive feed back mechanism as suggested for other vertebrates (Dickerman et al., 1972). Indeed, exogenous administration of estradiol and T elevate basal levels of GH in goldfish after 5 days of treatment (V. Trudeau, personal communication). However, whether increased levels of gonadal steroids have similar effects on GH release in pheromone-exposed males remains to be determined.

Gonadal Steroids and Milt production

The marked increases in both 17,20B-P and T observed in the blood of males exposed to water-borne 17,20B-P appear to be induced by the GtH responses to 17,20B-P, since increased levels of steroids are associated with elevated levels of GtH in goldfish and other teleosts (Kagawa et al., 1983; Ueda et al., 1983; Ueda et al., 1985; Kobayashi et al., 1986a). The rapid (within 15 min) changes in blood levels of 17,20B-P in the present study are consistent with those observed in earlier work (Dulka et al., 1987a; Chapter 2). However, unlike pheromone-induced increases in 17,20B-P, T increases have a latency of approximately 2 h.

The fact that plasma levels of 17,20B-P increase more rapidly than those of T in pheromone-exposed males is consistent with the proposal (Ueda et al., 1985) that 17,20B-P mediates the action of GtH on milt production in goldfish and salmonids. Although circulating levels of 17,20B-P are generally undetectable outside the spawning season, peak plasma levels of 17,20B-P coincide with periods of active spermiation in goldfish and a variety of other teleosts (Ueda et al., 1983, 1984, 1985; Scott and Baynes, 1982; Kobayashi et al., 1986b). Increased levels of GtH are believed to shift testicular biosynthesis from androgen to progestogen production through the suppression of the enzyme C21-C19 desmolase and the activation of 20B-hydroxysteroid dehydrogenase (Scott and Baynes, 1982). If true, one would expect levels of T to decrease as those of 17,20B-P increase in the blood of males exposed to the pheromone. However, in the present study, exposure to water-borne 17,20B-P caused increases in both 17,20B-P and T. One possible explanation for this finding is that high levels of GtH may initially stimulate the production of many testicular steroids and only after extended periods of time (greater than 12 h) would one expect to observe a biosynthetic shift from androgen to progestogen production. Conversely, male goldfish may not show a pronounced shift in steroid biosynthesis in response to high levels of GtH. For example, blood GtH, T, 11-ketotestosterone (11-KT), and 17,20B-P all are elevated during spawning in male goldfish (Kobayashi et al., 1986b). As suggested by others (Ueda et al., 1984, 1985; Scott and

Baynes, 1982; Kobayashi et al., 1986c), T and 11-KT may be involved with maintaining spermatogenesis rather than spermiation (i.e. milt production).

The GtH and steroid responses to water-borne 17,20B-P generally increase milt production within 4-6 h of exposure. Since preovulatory females begin to release water-borne 17,20B-P approximately 8-10 h prior to ovulation (Stacey et al., 1989), the latency of the milt response allows males to significantly increase milt production prior to spawning. However, under simulated natural conditions, males increase milt production earlier than would be predicted by the present results (Stacey et al., 1989). Although the reason for this is unknown, it is possible that preovulatory females release sufficient, but transient, amounts of water-borne 17,20B-P which stimulate males before increased levels of 17,20B-P can be measured in the water. It is also possible that 17α,-hydroxyprogesterone (17P), which is released by ovulatory goldfish (Van Der Kraak et al., 1989), and detected by 17,20B-P olfactory receptors (Sorensen et al., 1989), is released earlier in the preovulatory period than 17,20B-P.

In summary, exposure of male goldfish to water-borne 17,20B-P stimulates an increase in GtH which commences within 15 min, persists for at least 8-12 h, and causes an increase in milt production within 4-6 h. Changes in serum GH concentrations occurred inconsistently, and only after the increase in milt volume had occurred. Pheromone-induced increases in GtH stimulate the production of testicular 17,20B-P within 15 min and T within 2 h. Increased levels of testicular 17,20B-P, but not T, are believed to mediate the action of GtH on milt production. The latency of the endocrine and milt responses to water-borne 17,20B-P allows males to increase milt production prior to ovulation and spawning. I propose that the 17,20B-P pheromone system assists in the synchronizing the final stages of egg and sperm production in goldfish. This pheromone system appears to be the only vertebrate model in which the identity, cellular source and release of the pheromone, as well as the sensory pathway, endocrine mediators and the significance of the response in the pheromone recipient, all are clearly established. In addition, because male goldfish appear to respond to water-borne 17,20B-P in a reflexive manner, the 17,20B-P pheromone system serves as an excellent vertebrate model to study the neuroendocrine

regulation of endocrine responses to sex pheromones, as well as changes in testicular function during periods of "natural" sperm production.

Figure 5.1A-D. Time-course of serum GtH (**A**), GH (**B**), T (**C**) and milt volume (**D**) changes in male goldfish following exposure to either 100 µl ethanol (EtOH, clear bars) or $5X10^{-10}$ M water-borne 17,20B-P (shaded bars) for the times indicated. T concentrations were measured from the same blood samples used for determination of GtH and GH concentrations. n=12 in all groups. a=$p<0.05$ compared to 15 min EtOH. b=$p<0.05$ compared to 2 h EtOH group. c=$p<0.05$ compared to all EtOH groups.

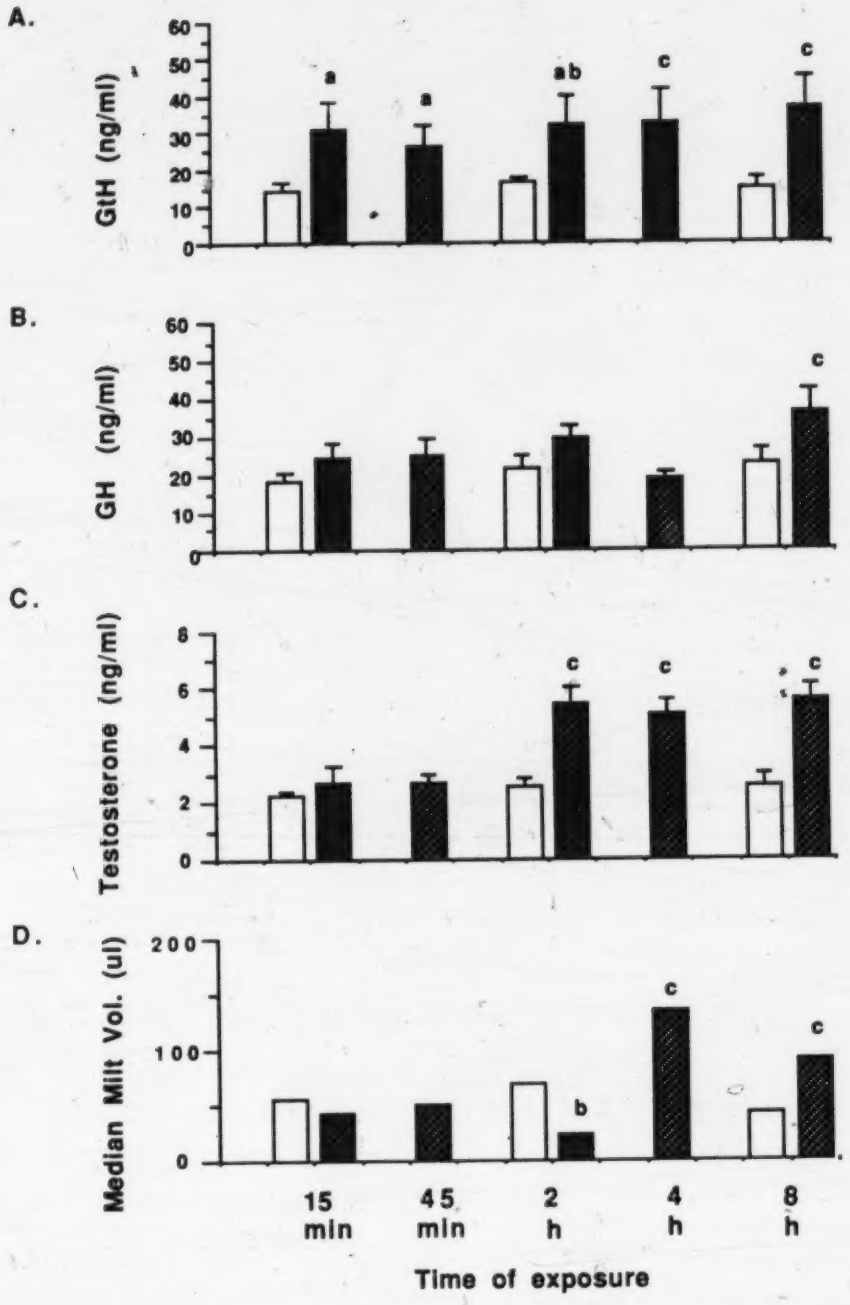

A.
GtH (ng/ml)
60
50
40
30
20
10
0
a
a
ab
c
c
B.
GH (ng/ml)
60
50
40
30
20
10
0
c
C.
Testosterone (ng/ml)
8
6
4
2
0
c
c
c
D.
Median Milt Vol. (ul)
200
100
0
b
c
c
15 min
45 min
2 h
4 h
8 h
Time of exposure

Figure 5.2A-D. Time-course of changes in serum levels of GtH (**A**), GH (**B**), 17,20B-P (**C**) and milt volume (**D**) in male goldfish following exposure to either 100 µl of ethanol (clear bars) or $5X10^{-10}$ M water-borne 17,20B-P for the times indicated. a=$p<0.05$ compared to the 30 min ethanol-exposed group. b=$p<0.05$ compared to the 2 h ethanol-exposed group. c=$p<0.05$ compared to the 8 h ethanol-exposed group. n=12 in all groups.

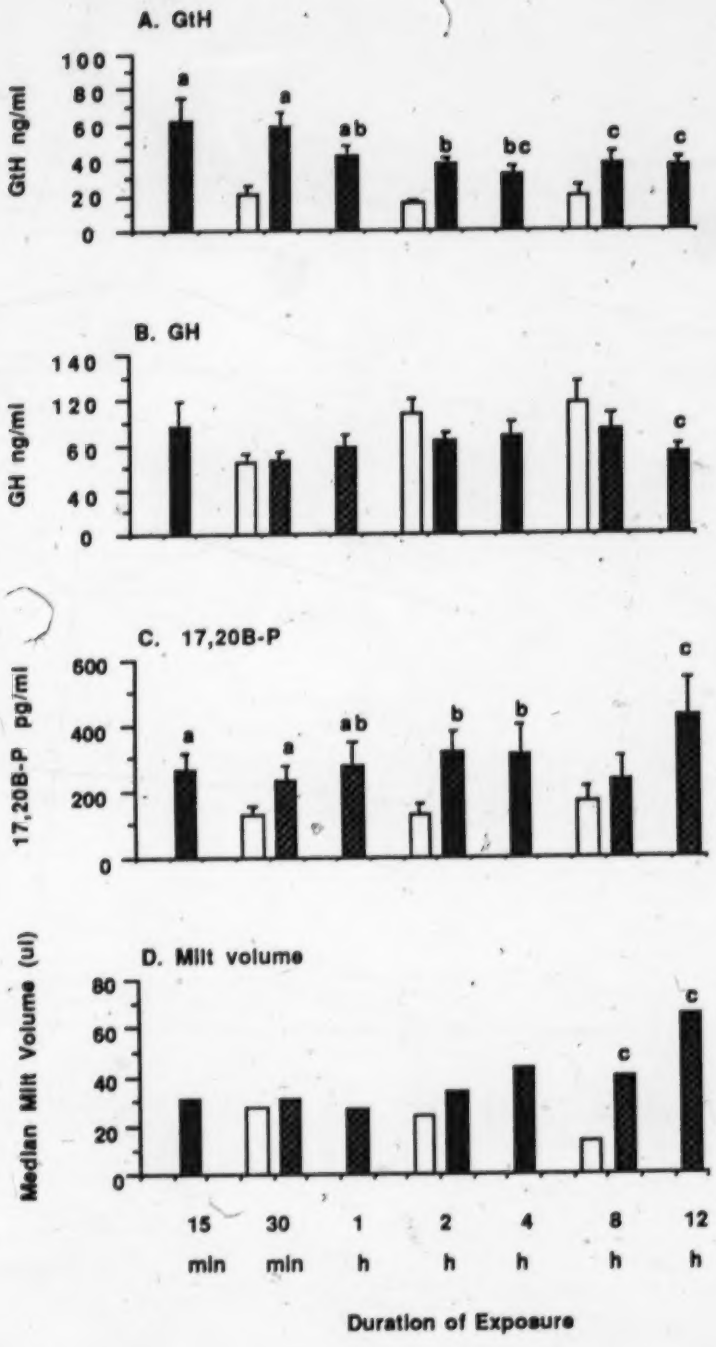

A. GtH
GtH ng/ml
100
80
60
40
20
0
a
a
ab
b
bc
c
c
B. GH
GH ng/ml
140
120
60
40
0
c
C. 17,20B-P
17,20B-P pg/ml
600
400
200
0
a
a
ab
b
b
c
D. Milt volume
Median Milt Volume (ul)
80
60
40
20
0
c
c
15 min
30 min
1 h
2 h
4 h
8 h
12 h
Duration of Exposure

Figure 5.3A-B. Milt volumes collected from male goldfish before (A) and after (B) exposure to either 100 µl of ethanol (EtOH) or $5X10^{-10}$ M water-borne 17,20B-P for the times indicated. Pre-exposure milt volumes in A were not significantly different. In B, * $p<0.05$ vs 6 h EtOH-exposed group, ** $p<0.01$ vs 8 h EtOH-exposed group. n=15 in all groups.

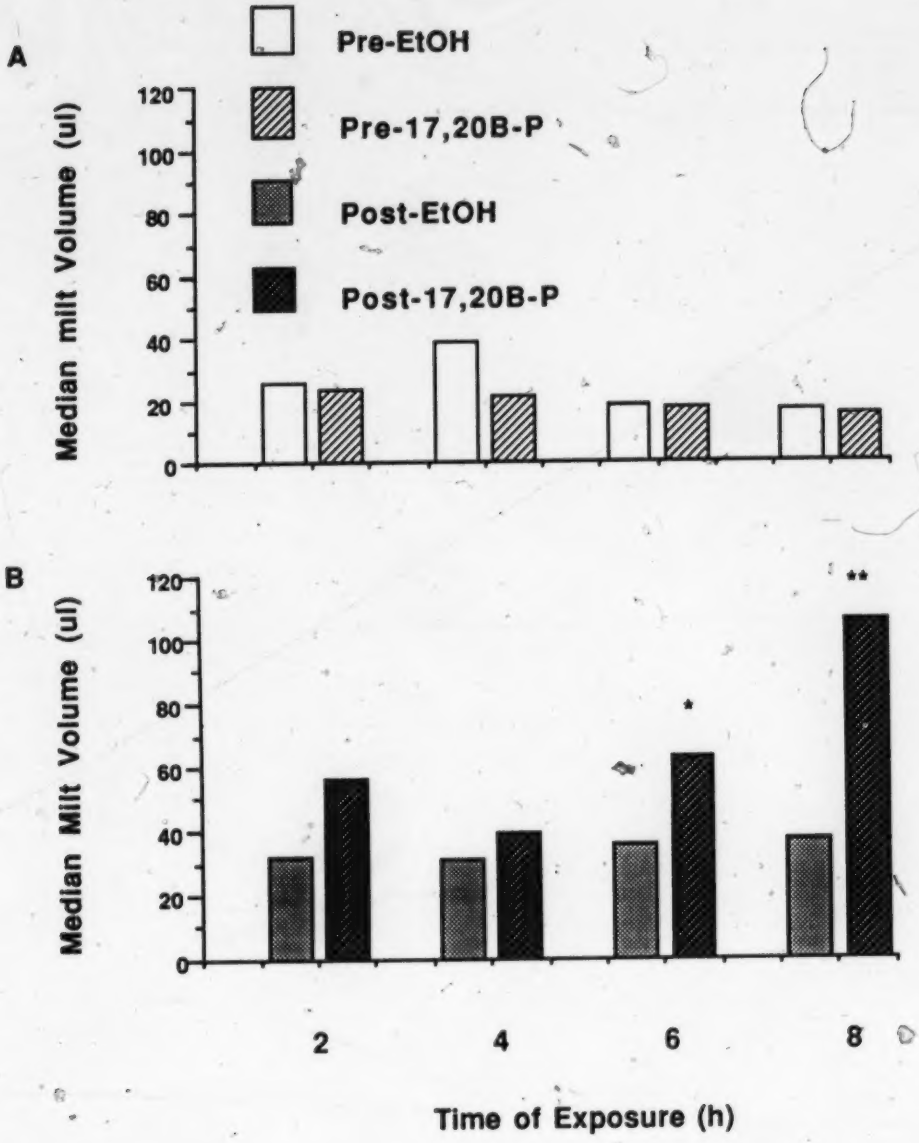
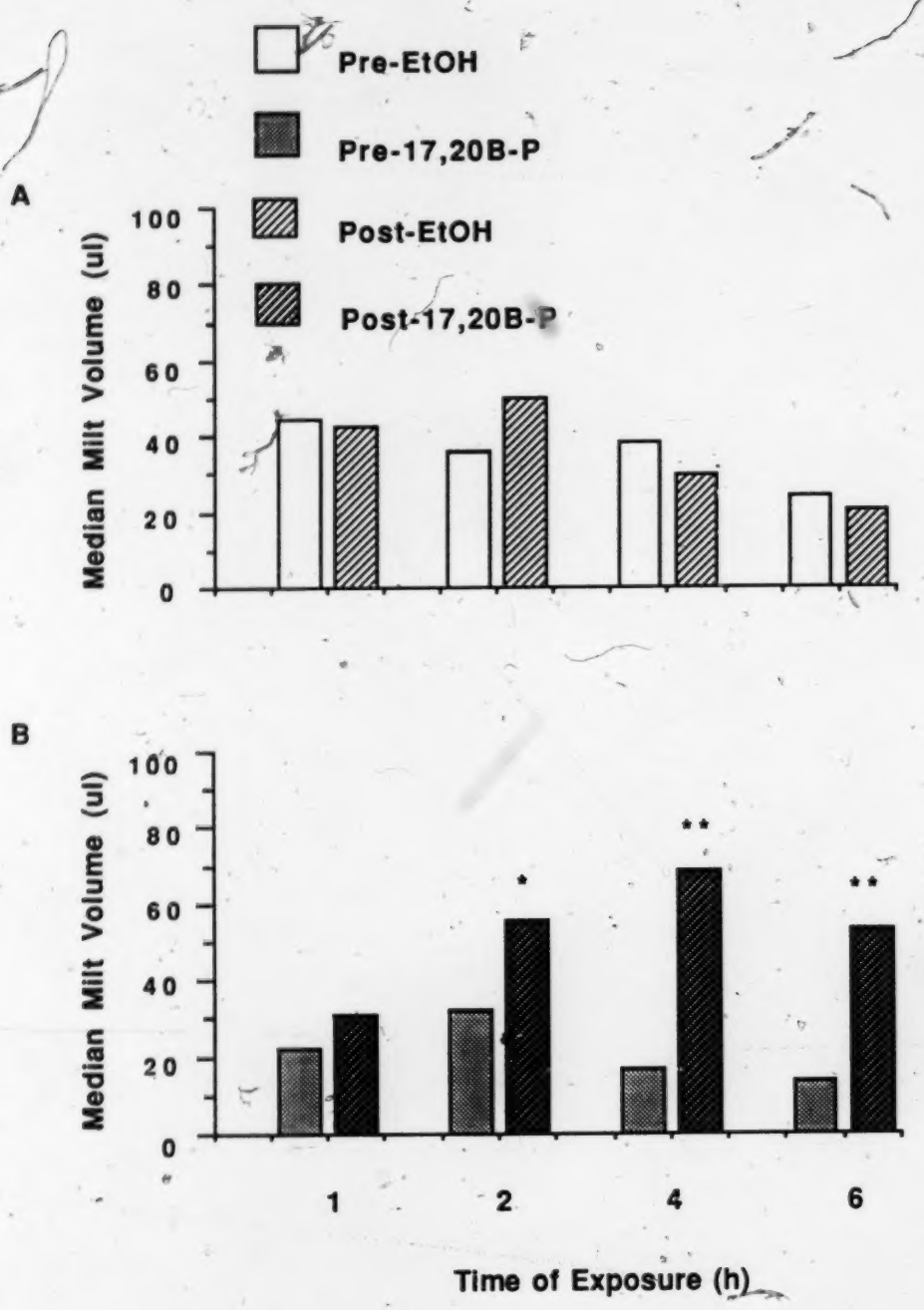

A
Pre-EtOH
Pre-17,20B-P
Post-EtOH
Post-17,20B-P
Median milt Volume (ul)
120
100
80
60
40
20
0
B
Median Milt Volume (ul)
120
100
80
60
40
20
0
**
*
2
4
6
8
Time of Exposure (h)

Figure 5.4A-B. Milt volumes collected from male goldfish before (**A**) and after (**B**) exposure to either 100 µl of ethanol (EtOH) or $5X10^{-10}$ M water-borne 17,20B-P for the times indicated. Pre-exposure milt volumes in **A** were not significantly different. In **B**, * $p<0.05$ and ** $p<0.01$ vs the EtOH exposed group at the same time. n=15 per group.


Pre-EtOH
Pre-17,20B-P
Post-EtOH
Post-17,20B-P
A
B
Median Milt Volume (ul)
100
80
60
40
20
0
*
**
**
1
2
4
6
Time of Exposure (h)

5.5 LITERATURE CITED

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6. CHARACTERIZATION OF MALE ENDOCRINE RESPONSES TO THE 17,20B-P FEMALE SEX PHEROMONE IN GOLDFISH: COMPARISON OF SCOTOPHASE AND PHOTOPHASE RESPONSIVENESS [1]

6.1 INTRODUCTION

Female goldfish release at least two sex pheromones during the periovulatory period (Sorensen and Stacey, 1989). The production and release of both pheromones are closely associated with the occurrence of a preovulatory surge of blood gonadotropin (GtH) which begins in the latter half of the photophase and induces ovulation, approximately 12 h later, in the latter half of the scotophase (Stacey et al., 1979).

Approximately 10 h prior to ovulation, female goldfish synthesize increased quantities of the steroid hormone, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), which induces final maturation of post vitelogenic oocytes (Goetz, 1983). 17,20B-P is then released to the water (Dulka et al., 1987a; Stacey et al., 1989) where it functions as a potent olfactory stimulant (Sorensen et al., 1987). Males exposed to water-borne 17,20B-P increase GtH within 15 min and milt volume within 4 h (Dulka et al., 1987a; Stacey et al., 1989; Chapter 5). Both the temporal pattern of 17,20B-P release from preovulatory females and the latency of the milt response in males allows milt production to be synchronized with ovulation (Dulka et al., 1987a). The 17,20B-P pheromone has minor behavioral effects on males, the function of which is unknown (Sorensen et al., 1989).

1. A version of this chapter has been published. Dulka, J.G., P.W. Sorensen, N.E. Stacey. 1987. *In*: Proceedings of the Third Symposium on the Reproductive Physiology of Fish. Edited by D.R. Idler, L.W. Crim and J.M. Walsh. Memorial Press. p. 160.

At the time of ovulation, females release a second pheromone which stimulates male sexual behavior. This pheromone is believed to be comprised of a mixture of at least two prostaglandins (PGs), prostaglandin $F_{2\alpha}$ (PGF) and 15-keto-prostaglandin $F_{2\alpha}$ (15K-PGF) (Sorensen et al., 1988). PGF functions endogenously in females to induce follicular rupture at ovulation and to stimulate female sexual behavior (Stacey and Goetz, 1982). The PGs are then released to the water as a pheromone which stimulates male sexual arousal and spawning activity (Sorensen et al., 1988). Females injected with PGF also release the PG pheromone to the water and become sexually attractive to males (Sorensen et al., 1988). Although the PG pheromone directly affects male behavior, males exposed to the odor of PG-releasing females show no change in blood GtH unless they are allowed to interact directly with conspecifics (Sorensen et al., 1988). In addition, males exhibit a marked circadian difference in endocrine responsiveness to PG-releasing females, such that GtH responses are maximal during the scotophase and reduced or absent during the photophase (Dulka et al., 1987b; N.E. Stacey, J.G. Dulka, P.W. Sorensen, unpublished results). However, because exposure to PG-releasing females stimulates male sexual behavior at all times (N.E. Stacey, unpublished results), the apparent day-night difference in GtH release in sexually active males appears to be due to a diurnal difference in endocrine responsiveness to the pheromone and not to differences in behavioral responsiveness.

Based on the premise that males would be most sensitive to 17,20B-P over the time period when the pheromone is normally released by females (22:00 h-06:00 h on a 16L:8D photoperiod in which photophase begins at 08:00 h; Stacey et al., 1989), all experiments described in Chapters 2-4 were conducted during the scotophase. The experiments described in this chapter examined whether males show a day-night difference in GtH responses to water-borne 17,20B-P. Experiment 1 compared the effects of 17,20B-P exposure at three times during the photophase and at one time during the scotophase. In Experiments 2 and 3, possible day-night differences in GtH responses to both the 17,20B-P pheromone and the PG pheromone were examined in parallel. These latter experiments were conducted at two times of the day, 12 h apart, such that the GtH responses to both

pheromones were examined during the photophase and scotophase.

6.2 MATERIALS and METHODS

Animals

Goldfish of the common or comet variety (20-30 g) were purchased at various times of the year from Ozark Fisheries Co. Stoutland, Missouri. Stocks of males were maintained in 1000 L flow-through aquaria at 18-20° C under a 16L:8D photoperiod (lights on at 08:00 h). Fish were fed Ewos trout pellets or Nutrafin flaked food *ad libitum* at least once a day. Only spermiated males with well developed pectoral fin tubercles were used in the experiments.

Experiment 1

This experiment was conducted in August, 1986, to compare male responsiveness to water-borne 17,20B-P at four times of the day. On day 1, groups of males were transferred to 65 L flow-through aquaria (3 fish/aquarium) at 20° C and randomly assigned to one of four treatment groups depending on the time of day they would be exposed to the pheromone. At either 10:00 h, 16:00 h, 22:00 h during the photophase on day 2, or at 04:00 h during the scotophase on day 3, the water flow to the appropriate aquaria was shut off. One half (n=15) of each treatment group was then exposed to 100 µl ethanol (control) while the other half was exposed to 10 µg 17,20B-P in 100 µl of ethanol. To promote rapid dilution in the water, the control and 17,20B-P solutions were injected directly into the column of air bubbles which originated from aeration stones in each aquarium. Although the resulting concentrations of 17,20B-P were not measured, the amount (10 µg) added to each aquarium should have created a final water concentration of $5X10^{-10}$ M.

After a 1 h exposure to the control and pheromone odors, the fish were anesthetized in a 0.05% solution of 2-phenoxyethanol (Syndel Laboratories, Vancouver, British Columbia), blood sampled through the caudal vasculature for determination of serum GtH

concentrations, and then returned to their respective aquaria. Eight hours later, the amount of milt which could be stripped from each fish was collected in pre-weighed hematocrit tubes which were then fitted with weighed caps as described previously (Stacey and Sorensen, 1986). The filled tubes were then weighed and the weight of milt calculated. Assuming a milt density of 1.0, the milt values are expressed as volumes (µl) rather than weights. The experiment was conducted in three separate runs over a 9 day period. Each run contained one third of the sample size for each treatment group. All aquaria were drained and rinsed thoroughly with flowing water for at least 24 h before the next run was conducted.

Experiment 2

This experiment was conducted in September, 1986, on the same stock of males used in experiment 1. The experiment examined male responsiveness to 17,20B-P at two times of the day, such that the fish were tested 12 h apart during the photophase (16:00 h) and scotophase (04:00 h). At both times, male GtH responses to 17,20B-P were compared to the GtH levels of males allowed to court and spawn with PG-injected females.

On day 1, males were transferred to test aquaria (3 fish/aquarium) at 20° C and divided equally into photophase (16:00 h) and scotophase (04:00 h) groups. One half of all aquaria contained floating artificial vegetation (planted) to serve as a spawning substrate (Stacey, 1981). The aquaria in which males would be exposed to ethanol or 17,20B-P lacked artificial vegetation (non-planted). At 16:00 h on day 2, the water flow to the appropriate aquaria was shut off. One half of the photophase males in non-planted aquaria were then exposed to 100 µl ethanol while the other half received 10 µg 17,20B-P as described above. During this same period, half of the planted aquaria each received two females which had been injected (im.) with PGF (300 ng/g, 5.0 µl/g body wt.) and the other half each received two saline-injected females. All injections took place immediately before the females were added to the aquaria. After 1 h exposure to the above treatments, the males were anesthetized and blood sampled for determination of GtH concentrations.

Treatment effects on milt volume were not examined in this experiment. During the 1 h exposure period, the males in contact with PG-injected females were observed to verify whether they were performing courtship and/or spawning behaviors. This entire procedure was repeated at 04:00 h on the scotophase group of males. Scotophase males were briefly observed under dim illumination to check for spawning activity. The experiment was conducted in two runs over a 5 day period; each run consisting of one half of the sample size for each treatment.

Experiment 3

This experiment was conducted in July, 1987, on a new stock of males. The procedures employed in this experiment were similar to those described for Experiment 2, except for the following modifications. On day 1, males were transferred to planted test aquaria and randomly divided into photophase (16:00 h) and scotophase (04:00 h) groups. Both the photophase and scotophase males were then divided into 4 groups which received the following treatments on day 2 (photophase), or day 3 (scotophase). The first group of males was exposed to 100 µl ethanol alone. The second group was exposed to 100 µl ethanol and 2 saline-injected females. The third group was exposed to 10 µg 17,20B-P and two saline-injected females. The fourth group was exposed to 100 µl ethanol and 2 PG-injected females. All males in contact with PG-injected females were observed to verify spawning activity. Following a 1 h exposure to the above treatments, the males were anesthetized and blood sampled for determination of serum GtH concentrations. Milt was not stripped from fish in this experiment. The experiment was conducted in a single run over a 3 day period.

Hormone Assays

Blood samples were allowed to clot on chipped ice before being centrifuged to obtain serum which was then stored frozen until assayed. Serum GtH concentrations were determined by radioimmunoassay (RIA) as described previously (Peter et al., 1984).

Statistical Analysis

All data were analysed for homogeneity of variances using Bartlett's tests. Data not normally distributed were transformed before being analysed by parametric 2-way analysis of variance (ANOVA).

In Experiment 1, GtH values were log transformed and analysed by 2-way ANOVA. Comparisons between ethanol and 17,20B-P-exposed groups at each sample time were performed by Least Squares Means analysis using a SAS computer package (SAS Institute Inc., Cary, NC). Comparisons between 17,20B-P- and ethanol-exposed groups at different sample times were performed using Duncan's multiple range tests. The milt volume data from both experiments were square root transformed and analysed by 2-way ANOVA. Comparisons between ethanol and 17,20B-P groups at each sample time were performed by Least Squares Means analysis.

In Experiment 2, the groups exposed to ethanol and 17,20B-P and those exposed to non-injected and PG-injected females were analysed separately. In both cases, the GtH values were log transformed and analysed by 2-way ANOVA. Most differences between treatment groups were determined by Duncan's multiple range tests. However, differences between control males exposed to ethanol and control males exposed to saline-injected males were analyzed by t-tests for *a posteriori* comparisons (Marascuilo and Sweeney, 1977).

All data from Experiment 3 were log transformed and analysed by 2-way ANOVA. Comparisons between treatment groups were performed by Duncan's multiple range tests.

6.3 RESULTS

Experiment 1

Serum GtH levels in ethanol-exposed controls were similar at all times of the day (Fig. 6.1A). Males exposed to 17,20B-P had significantly ($p<0.01$) higher levels of GtH than ethanol-exposed controls at all sample times. In addition, the magnitudes of the GtH responses were similar in all 17,20B-P-exposed groups, regardless of the time of exposure (Fig. 6.1A).

The treatment effects on milt volume were similar to those on GtH (Fig. 6.1B). Control animals exposed to ethanol had equivalent milt volumes at all times of the day. 17,20B-P exposure significantly ($p<0.05$) elevated milt volume in males exposed to the pheromone at 10:00 h, 16:00 h, and 22:00 h (Fig. 6.1B). Although males exposed to 17,20B-P at 04:00 h had higher milt volumes than fish exposed to ethanol, the difference between these two groups was not significant.

Experiment 2

GtH levels in the two control groups (exposure to ethanol in non-planted aquaria; exposure to non-injected females in planted aquaria), were similar during the scotophase and photophase, respectively (Fig. 6.2). However, the GtH levels of female-exposed controls (planted aquaria) were significantly ($p<0.05$) higher than those of ethanol-exposed controls (non-planted aquaria) at both sample times (Fig. 6.2). Compared to the males exposed to ethanol, males exposed to 17,20B-P had significantly ($p<0.05$) higher serum GtH levels during both photophase and scotophase. In contrast, although males actively chased PG-injected females during both the scotophase and photophase, exposure to PG-injected females was effective at increasing ($p<0.05$) male GtH only during the scotophase (Fig. 6.2).

Experiment 3

Males exposed to ethanol alone or ethanol and 2 saline-injected females had low and comparable levels of GtH during the photophase and scotophase (Fig. 6.3). At both sample times, males exposed to 17,20B-P had significantly ($p<0.05$) higher GtH levels than males exposed to ethanol. In contrast to the previous experiment, males exposed to PG-injected females had higher ($p<0.05$) levels of GtH than males exposed to saline-injected females during both the photophase and scotophase (Fig. 6.3).

6.4 DISCUSSION

The results of the present study clearly indicate that at all times of the day male goldfish are capable of increasing serum GtH when exposed to water-borne 17,20B-P. In addition, Experiment 1 failed to demonstrate a circadian change in the magnitude of the male GtH response to 17,20B-P in that the pheromone was no more effective at increasing male GtH during the scotophase than it was during the photophase. In Experiments 2 and 3, exposure of males to 17,20B-P induced an equivalent increase in GtH during both photophase and scotophase, consistent with the findings of Experiment 1. In Experiment 2, exposure of males to PG-injected females increased male GtH only during the scotophase, an effect which has been seen in other work (Dulka et al., 1987b; N.E. Stacey, P.W. Sorensen, J.G. Dulka, unpublished results). Increased nocturnal sensitivity to PG-injected females may be biologically relevant since males also show a similar day-night difference in GtH release when exposed to ovulating females (N.E. Stacey, unpublished results). In contrast, the results from Experiment 3, as well as those of other studies (Kyle et al., 1985; Sorensen et al., 1989) demonstrate a stimulation of male GtH release following exposure to PG-injected females during the photophase. The GtH response to PG-injected females, however, does not appear to be due to the direct actions of the PG pheromone. For example, whereas isolated males increase milt production in response to water-borne 17,20B-P (Stacey and Sorensen, 1986), isolated males show no change in circulating

levels of GtH when exposed to water-borne PGFs (Sorensen et al., 1989). In this regard, the PG pheromone is thought to indirectly influence GtH release by stimulating male sexual arousal (Sorensen et al., 1989; see Chapter 7). For example, increased levels of GtH occur when groups of males are exposed to mixtures of PGs which stimulate a high degree of chasing behavior among males as if they were searching for a female (Sorensen et al., 1988; Sorensen et al., 1989). Thus, social and/or sexual interactions appear to be prerequisites for the occurrence of male GtH responses to PG-injected females. The functional link between conspecific interaction and behaviorally-induced GtH release appears to be less pronounced during the photophase than during the scotophase. However, the mechanism controlling this difference in circadian sensitivity to PG-releasing females is not known.

The finding that males sometimes exhibit circadian differences in endocrine sensitivity to the stimulus of PG-females, but not to 17,20B-P, suggests that these two stimuli act through separate neuroendocrine mechanisms to regulate GtH release in males. Although the reason for this difference is not known, it may not be surprising that males lack a marked day-night difference in endocrine responsiveness to water-borne 17,20B-P, considering the extended time period (22:00-06:00 h) over which females normally release 17,20B-P (Stacey et al., 1989). Since the release of 17,20B-P encompasses both the photophase and scotophase, selection pressures may not have operated to restrict male responsiveness to one of the time periods.

In summary, GtH increases in the blood of male goldfish exposed to water-borne 17,20B-P and following behavioral interaction with PG-releasing females. The GtH response to 17,20B-P occurs consistently at all times of the day whereas the GtH response to PG-releasing females is often greatest during the scotophase. The results suggest that the GtH responses to these two forms of stimulation are regulated by separate neuroendocrine mechanisms. GtH responses to 17,20B-P, and perhaps to ovulated females at mid-scotophase, evidently function to increase milt production by the time of ovulation (Dulka et al., 1987a). However, it is not clear what reproductive function is served by GtH responses to PG-releasing females during photophase.

Figure 6.1. Serum GtH levels (**A**) and milt volumes (**B**) in male goldfish following a 1 h exposure to either 100 µl ethanol (clear bars) or $5X10^{-10}$ M 17,20B-P (shaded bars) at four times of the day. Significant differences between ethanol and 17,20B-P exposed groups at the same time are indicated by an asterisk; *$p<0.05$; ** $p<0.01$. For GtH, comparisons between groups exposed at different times are indicated by the italicized letters at the upper right hand corner of the figure. Groups underlined by the same line are not significantly different ($p>0.05$). All statistical analysis was performed by 2-way ANOVA and Least Squares Means analysis. n=15 in all groups.

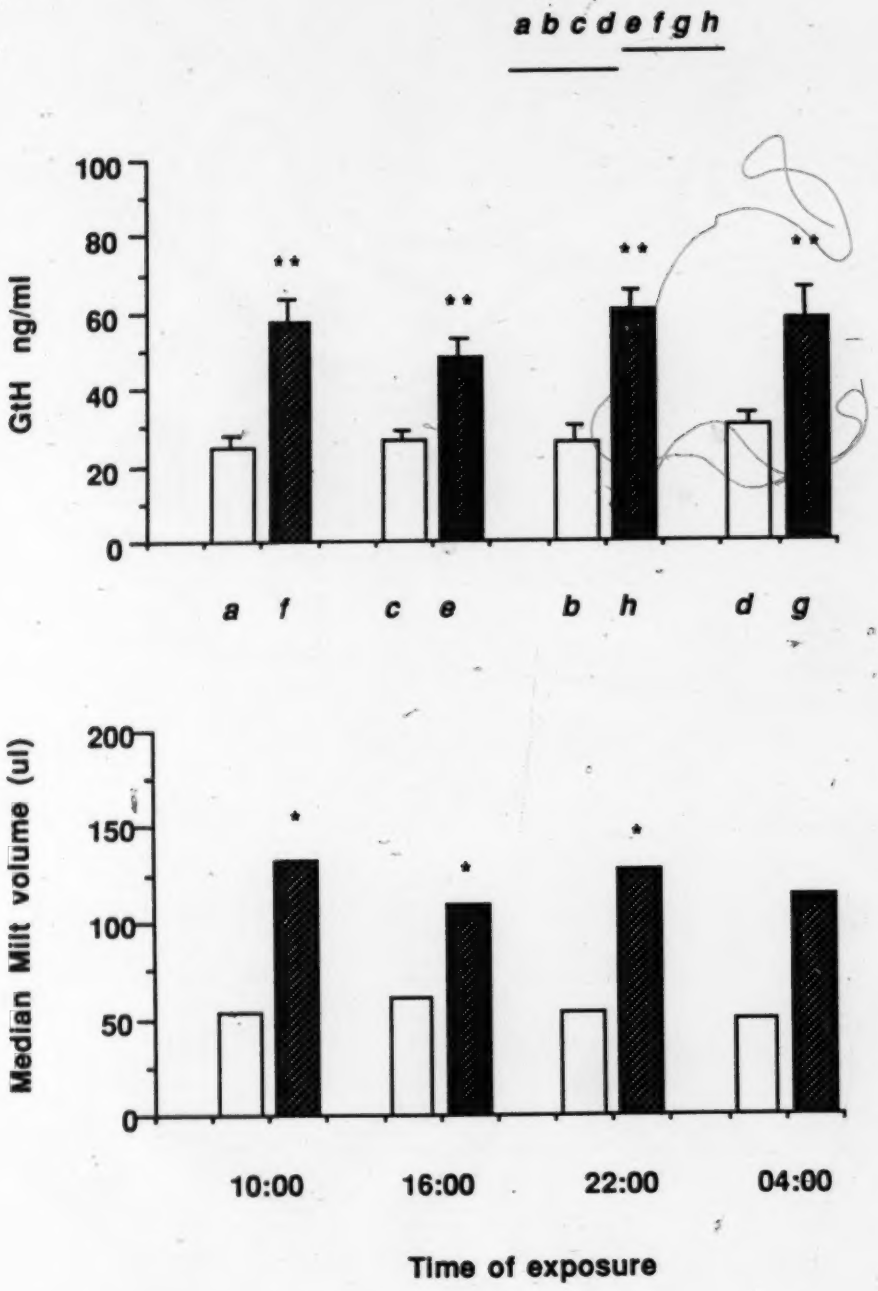
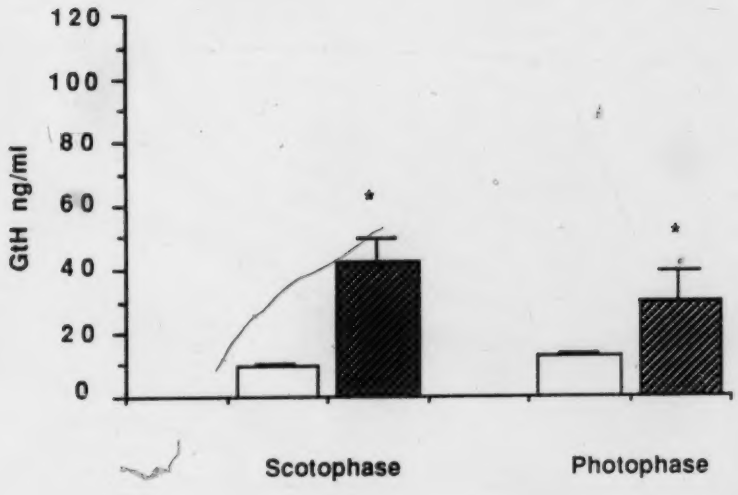

a b c d e f g h
GtH ng/ml
100
80
60
40
20
0
**
**
**
**
a f c e b h d g
Median Milt volume (ul)
200
150
100
50
0
*
*
*
10:00
16:00
22:00
04:00
Time of exposure

Figure 6.2. (**A**) Serum GtH levels in male goldfish following a 1 h exposure to 100 µl ethanol (clear bars) or $5X10^{-10}$ M water-borne 17,20B-P (Shaded bars) during either the scotophase or photophase. (**B**) Serum GtH levels in male goldfish following a 1 h exposure to two non-injected females (lightly shaded bars) or two PG-injected females (darkly shaded bars) during either the scotophase or photophase. Significant differences between ethanol and 17,20B-P-exposed groups, and between non-injected female and PG-injected female-exposed groups are indicated by an asterisk ($p<0.05$, Least Squares Means analysis). n=12 in all groups.

A. 17,20B-P



B. SPAWNING

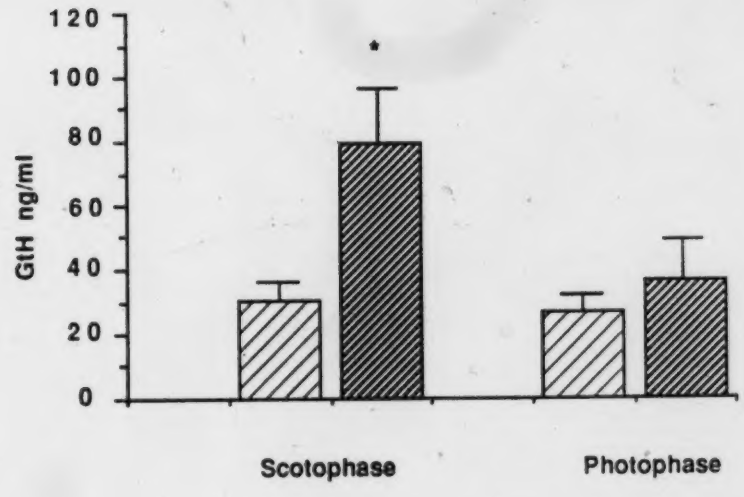
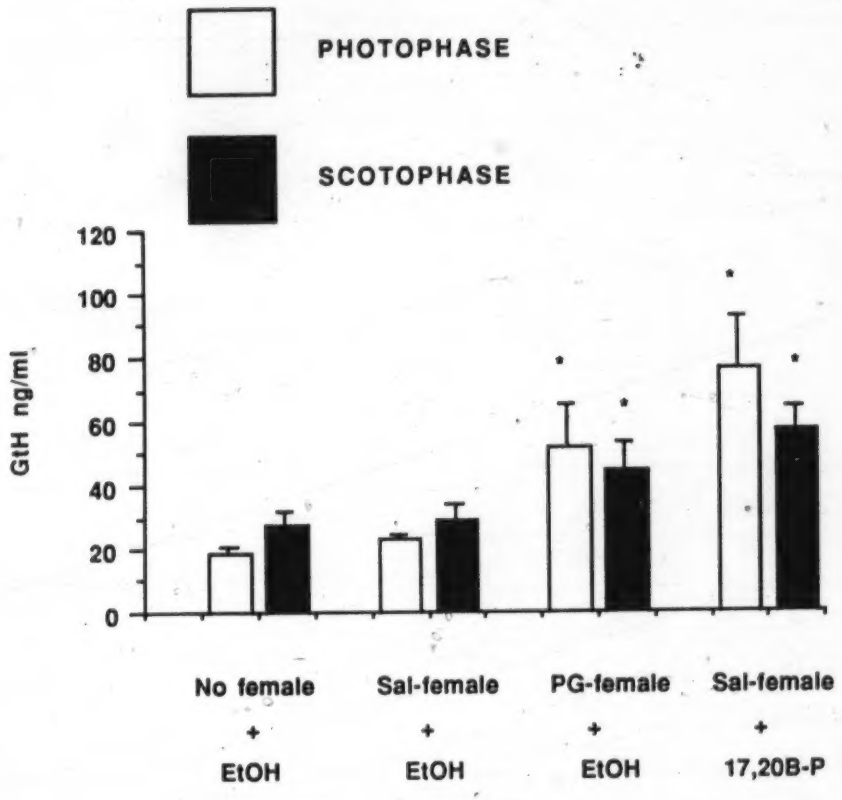


Figure 6.3. Serum GtH levels in male goldfish following a 1 h exposure to the indicated treatments during either photophase or scotophase. Significant differences between groups are indicated by an asterisk ($p<0.05$, Least Squares Means analysis). n=8 for the group exposed to ethanol alone; n=16 in all other groups.



* p<0.05 compared to Sal-female+EtOH group at the same time

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7. SEX PHEROMONE RELEASE FROM FEMALE GOLDFISH AND ASSOCIATED CHANGES IN GONADOTROPIN AND GROWTH HORMONE SECRETION IN MALES: EFFECTS OF PREOVULATORY AND POSTOVULATORY PHEROMONES [1]

7.1 INTRODUCTION

Pheromones are considered to have either *primer* or *releaser* effects on conspecifics (Wilson and Bossert, 1963). In general, *primer* effects involve physiological processes, independent of behavior, whereas *releaser* effects involve stereotyped behavioral responses. It has been recently demonstrated that ovulatory female goldfish release both preovulatory *primer* (Dulka et al., 1987a; Stacey et al., 1989) and postovulatory *releaser* pheromones (Sorensen et al., 1988) which act as potent olfactory stimulants in males (Sorensen et al., 1987; Sorensen et al., 1989). In goldfish, the proposed maturation-inducing steroid hormone, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P; Nagahama, 1983), exerts potent pheromonal *primer* effects on males by stimulating rapid increases in gonadotropin (GtH) secretion and a consequent increase in milt (sperm and seminal fluid) production (Stacey and Sorensen, 1986; Dulka et al., 1987a). 17,20B-P also acts as a *releaser* to evoke subtle behavioral changes in males (Sorensen et al., 1989); however, the function of these behavioral responses is not known. The postovulatory *releaser* pheromone in goldfish, which stimulates male sexual behavior, appears to be comprised of

1. A portion of this chapter has been submitted for publication. Sorensen, P.W., N.E. Stacey, and K.J. Chamberlain. 1989. Horm. Behav.

a mixture of at least two prostaglandins (PGs), prostaglandin $F_{2\alpha}$ (PGF) and its metabolite 15-keto-prostaglandin $F_{2\alpha}$(15K-PGF) (Sorensen, et al., 1988). Females injected with PGF release increased levels of these PGs to the water (Sorensen et al., 1986; 1988) and become both sexually active and attractive to males (Stacey and Goetz, 1982). In addition, males which spawn with PG-injected females can increase GtH and milt volume within 20 min. (Kyle et al., 1985). However, isolated males exposed to water-borne PGs, in the absence of females, do not exhibit increases in GtH and milt (Sorensen et al 1989). Sorensen et al., (1989) recently compared the effects of 17,20B-P and PGs on male GtH levels and concluded that at least two controlling mechanisms are involved, one involving direct *primer* actions of 17,20B-P, the second being mediated, indirectly, through behavioral *releaser* actions of water-borne PGs.

This study sought to further examine the direct and indirect actions of these pheromones by monitoring not only GtH but also growth hormone (GH) release under conditions in which males would be expected to detect either water-borne 17,20B-P or PGs. GH release in males was examined for two reasons. First, although female goldfish show simultaneous increases in both GtH and GH during ovulation (Marchant, 1983), the pattern of GH release in males during this period has not been elucidated. Second, preliminary findings indicate that water-borne 17,20B-P does not affect GH release in males (Dulka et al. 1987b; Chapter 4); however, it remained unclear whether males exposed to water-borne PGs would show increases in both hormones, as is the case with ovulatory females.

Since increased synthesis and release of both 17,20B-P and PGs are directly associated with the ovulatory GtH surge, I sought to increase the production of these two pheromones by injecting females with human chorionic godadotropin (hCG). hCG treatment stimulates ovarian production of 17,20B-P which causes final oocyte maturation and leads to ovulation within 10-12 h at 20^0 C (M. Kobayashi, personal communication). Follicular rupture at the time of ovulation and the activation of female sexual behavior are both believed to be triggered by increased synthesis of PGs (Stacey and Goetz, 1982;

Goetz et al. 1987) which are then released to the water as pheromones (Sorensen et al., 1988). The periovulatory profiles of 17,20B-P and PGs released by spontaneously ovulating females do not overlap. Increased release of 17,20B-P begins approximately 7-10 h prior to ovulation, peaks within 1-4 h prior to ovulation and then rapidly declines by the time females ovulate (Stacey et al. 1989). PG release to the water begins to increase at the time of ovulation and continues to increase for at least 6 h, provided that ovulated eggs remain in the reproductive tract (Sorensen et al., 1988). Based on other studies (M. Kobayashi, personal communication), it is likely that a similar release pattern of the two pheromones would occur in hCG-treated females. Thus, males placed with preovulatory hCG-treated females should be exposed to water-borne 17,20B-P, whereas males placed with postovulatory hCG-treated females should be exposed to water-borne PGs.

In this study, the serum GtH and GH levels of males exposed to preovulatory hCG-treated females were examined when peak 17,20B-P release was expected to occur and compared to those responses observed following addition of 17,20B-P to the water. The influence of behavioral interactions with females on male GtH and GH was examined by exposing males to ovulated hCG-treated and PG-injected females. The direct actions of PGs on GtH and GH release in males was examined by exposing isolated males to water-borne PGs alone.

7.2 MATERIALS AND METHODS

Animals:

Goldfish of the common or comet variety (20-30 g) were purchased from Ozark Fisheries Co. Stoutland, Missouri. Sexually mature males having well developed pectoral tubercles (pearl organs) and expressible milt were maintained in 1000 L flow-through stock aquaria at 15-20° C under a 16L:8D photoperiod (lights on at 08:00 h). Fish were fed Ewos trout pellets and Nutrafin flaked food *ad libitum* at least once a day.

Experiment 1: Male GtH and GH responses to preovulatory hCG-injected females.

This experiment determined whether males exhibit increased GtH and/or GH following exposure to preovulatory females which are known to release high levels of 17,20B-P (Dulka et al., 1987; Stacey et at., 1989). Groups of males were placed in 65 L flow-through aquaria (3 fish/aquarium, 20° C) containing artificial vegetation and gravel substrate one day before the experiment. At 20:00 h on the following day, one sexually mature female was injected intraperitoneally (i.p.) with either saline (5 µl/g) or hCG (2.5 U/g, 5.0 µl/g) and added to each aquarium. At 04:00 h the following morning, all fish were anesthetized in a 0.05% solution of 2-phenoxyethanol (Syndel) and blood sampled from the caudal vasculature. Females were checked for ovulation at the time of blood sampling and again at 08:00 h and 10:00 h, when all females were sacrificed to determine the degree of oocyte maturation as described by Jalabert et al. (1978).

Experiment 2: Male GtH and GH responses to water-borne 17,20B-P.

This experiment examined direct effects of water-borne 17,20B-P on GtH and GH release in male goldfish. Groups of males were placed in 65 L flow-through aquaria (3 fish/aquarium, 20° C) one day before the experiment. Artificial vegetation was not present in the aquaria. On the day of the experiment, the water flow to the aquaria was shut off and either 100 µl of ethanol or 10 µg 17,20B-P in 100 µl of ethanol (final water concentration approximately $5X10^{-10}$ M) was added to the water using a glass syringe. All males were blood sampled after 1 h of exposure to ethanol or 17,20B-P.

Experiment 3: Male GtH and GH responses to postovulatory hCG-injected females.

This experiment examined whether males exhibit increased GtH and/or GH following exposure to postovulatory females which are known to release increased levels of PGs (Sorensen et al., 1988). The aquaria in this experiment were divided transversely by an opaque barrier which allowed water exchange between compartments (Stacey et al., 1989). Each aquarium contained gravel and aquatic vegetation on both sides of the barrier. An

airstone was present in one compartment to promote water circulation throughout the aquarium. On the day before the experiment, individual males were placed on one side of the barrier in each of the aquaria. At 10:00 h the following day (day 2), the males then received one of 4 treatments: 1. the individual male remained isolated (no additional fish or fish odors were added to the aquarium, ISOL); 2. a saline-injected, nonovulated, female was added to the compartment opposite the male (NONOV-WATER); 3. an hCG-injected female which had ovulated within several hours was added to the compartment opposite the male (OV-WATER); 4. an hCG-injected ovulated female was placed in the compartment containing the male (OV-CONT). Ovulated females in treatments 3 and 4 were injected with hCG (i.p., 2.5 U/g, 5.0 µl/g) 12 h before being checked for ovulation. To reduce the possibility that recently ovulated females might contaminate experimental aquaria with 17,20B-P, the females were transferred from ovulation aquaria to holding aquaria for at least 1 h before being rinsed in fresh water and added to aquaria containing males. 17,20B-P release to the water, however, was not measured in this experiment. Each treatment lasted for 1-1.5 h (09:00-12:00 h) following which the males were anesthetized and blood sampled.

Experiment 4: Male GtH and GH responses to postovulatory, hCG-injected and PG-injected females.

This experiment examined whether the GtH and GH levels of males exposed to PGF-injected females are similar to those observed following exposure to ovulated females. Individual males were placed in aquaria containing artificial vegetation and gravel substrate one day before the experiment. On the following morning, either a PG-injected (1.0 µg PGF/µl saline buffer, 3.0 µl/fish, im.) female, a saline-injected (3.0 µl saline buffer/fish) female or a female induced to ovulate by hCG injection was added to each aquarium. The procedure for inducing ovulation with hCG was identical to that described for Experiments 1 and 3 (see above). PG-injected females were placed in separate aquaria for 15 min before being added to aquaria containing males. Males exposed to PG-injected or ovulated females were observed to ensure that they spawned during the test period. Each treatment lasted for

1.5 h, after which the males were anesthetized and blood sampled.

Experiment 5: Endocrine responses of isolated males to preovulatory and postovulatory sex pheromones.

This experiment examined endocrine responses to the 17,20B-P and PG pheromones in individual males to determine whether behavioral interactions with females are necessary for GtH responses to water-borne PGs. Individual males were placed in aquaria (as described above) one day before the experiment and randomly assigned to one of 8 groups. Four groups were exposed to different pheromone solutions which were added directly to the water using a peristaltic pump. These included a solution of 17,20B-P ($5X10^{-7}$ M), a mixture of PGF and 15K-PGF ($5X10^{-7}$ M), holding water from a PG-injected female and an ethanol (CON1) control solution. Stock solutions of 17,20B-P (Sigma), PGF and 15K-PGF (Cayman Chemical Co., MI) were made in 95% ethanol at 1.0 mg/ml. Test solutions were diluted immediately before use by adding the appropriate amount of each stock solution or ethanol to distilled water. PG-injected female water was prepared by injecting a female with PGF (see above) and placing it in 1 L of aquarium water 15 min before being used in the experiment. All pheromone solutions were pumped into aquaria at a rate of 10 ml/min as described previously (Sorensen et al., 1989). The remaining 4 groups of males received one of the following treatments: either a PG-injected female, a saline-injected female, a few grams of food (Tetramin flaked food) or nothing (CON2) was added to the aquaria. All treatments lasted for 1 h after which the males were anesthetized and blood sampled. The GtH data from this experiment have been published elsewhere (Sorensen et al. 1989).

Hormone assays:

GtH and GH levels in male and female blood and 17,20B-P levels in female blood were determined by radioimmunoassay (RIA) as described previously (Peter et al., 1984; Cook et al., 1983; Scott et al., 1982; Van Der Kraak et al., 1984).

Statistical analysis:

In Experiment 1, female blood 17,20B-P values were log transformed and analyzed with a one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple range tests. Male GtH and GH values were log transformed and analyzed with ANOVA; differences between groups were determined by Least Squares Means analysis (Barr et al. 1979). In all other experiments, male GtH and GH values were log transformed and analyzed with ANOVA and Student-Newman-Keuls multiple range tests.

7.3 RESULTS

Experiment 1

Six of 30 hCG-injected females ovulated (OV), all between 09:00 and 11:00 h (i.e. 13 h to 15 h post-hCG injection). The remainder of the hCG-injected females were divided into two categories based on their degree of oocyte maturation. Eight females did not undergo oocyte final maturation maturation (DUD), the germinal vesicle remaining centrally located within the oocyte. Oocytes from DUD females appeared similar to those observed from saline-injected females (n=14). Sixteen females, did not ovulate but underwent oocyte maturation (MAT); the germinal vesicle had migrated from the center of the oocyte toward the periphery and in some cases had broken down.

At 04:00 h (i.e. 8 h post-hCG injection), blood levels of GtH in all four female groups were low indicating that spontaneous ovulations had not occurred (i.e. all 6 ovulations were induced by hCG-treatment, data not presented). Blood levels of 17,20B-P in MAT and OV females were similar and significantly ($p<0.01$) higher than levels of saline-injected controls and DUD females (Fig 7.1A).

Males exposed to saline-injected (n=44) and DUD females (n=27) had low and equivalent levels of blood GtH (Fig 7.1B). In contrast, males exposed to MAT (n=47) and OV (n=18) females had similar but significantly ($p<0.001$) higher GtH than males exposed

to saline-injected and DUD females. GH levels in males exposed to MAT and OV females were significantly ($p<0.05$) higher than those of males exposed to saline-injected females, but similar to those males exposed to DUD females (Fig 7.1C).

Experiment 2

Males exposed to 17,20B-P for 1 h had significantly ($p<0.001$) higher blood GtH than males exposed to ethanol (Fig 7.2A). GH levels in males exposed to 17,20B-P were not significantly different from ethanol-exposed controls (Fig 7.2B).

Experiment 3

GtH levels in males exposed to ovulated females (OV-CONT) were significantly greater than all other groups (Fig. 7.3A). Males exposed to OV-WATER had significantly elevated GtH compared to SAL controls, but not compared to NONOV-WATER males. Blood GH levels were similar in all groups (Fig 7.3B).

Experiment 4

Males exposed to saline-injected, nonovulatory females had low GtH levels which were similar to those of the above experiments (Fig 7.4A). Males exposed to ovulated and PG-injected females had equivalent and significantly higher GtH than males exposed to nonovulated females. Blood GH levels in males exposed to ovulated females and PG-injected females were not significantly different from those of males exposed to saline-injected females (Fig 7.4B).

Experiment 5

Males which remained isolated and those exposed to ethanol (CON1) had low and equivalent levels of GtH (Fig 7.5A). Individual males exposed to 17,20B-P, but not those exposed to the PG-mixture or PG-female water, had significantly ($p<0.05$) higher GtH than ethanol-exposed controls. Individual males in contact with saline-injected and PGF-injected females had significantly ($p<0.05$ and $p<0.01$, respectively) higher GtH levels

than isolated males (CON2). Males in contact with saline-injected and PG-injected females actively chased the females, but spawning was observed only in males exposed to PG-injected females. Exposure to food odor caused a nonsignificant increase in male GtH compared to that of isolated males. Blood GH levels were similar in all groups of males (Fig 7.5B).

7.4 DISCUSSION

Gonadotropin

The present results are consistent with our earlier studies (Stacey and Sorensen, 1986; Dulka et al. 1987a; Stacey et al. 1989) which show that 17,20B-P functions as a potent preovulatory *primer* pheromone that stimulates GtH release in males. In Experiment 1, blood levels of 17,20B-P were highest in preovulatory females which eventually underwent final oocyte maturation. Increased production of 17,20B-P by females, however, does not determine whether the female will eventually ovulate. For example, females which underwent oocyte final maturation, but failed to ovulate, not only had high plasma levels of 17,20B-P but also apparently released 17,20B-P to the water in amounts sufficient to induce a GtH response in males. Although 17,20B-P release to the water was not measured in this experiment, the results suggest that the stimulatory action of 17,20B-P on males is primarily associated with the period of oocyte final maturation. In support, plasma 17,20B-P in spontaneously ovulating goldfish quickly increases within the first half of the GtH surge and then rapidly decreases by the time of ovulation (Kobayashi et al. 1987; Stacey et al. 1989). Release of 17,20B-P to the water by preovulatory females begins within 3 h of the initiation of the GtH surge, peaks 1-4 h prior to ovulation and then dramatically declines to pre-surge levels by 2 h post-ovulation (Stacey et al. 1989). Detection of the pheromone by males stimulates GtH secretion and ultimately leads to increased milt production by the time of ovulation (Dulka et al. 1987a).

At the time of ovulation, female goldfish begin to release a second pheromone which

is comprised of PGF and/or its metabolites (Sorensen et al. 1988; Sorensen and Stacey, 1989). Although this postovulatory pheromone functions primarily as a *releaser* of male sex behavior, males which spawn with ovulated or PG-injected females exhibit rapid increases in serum GtH and milt volume (Kyle et al. 1985). Rapid increases in GtH also are observed when groups of males are exposed to PG-injected females or the odor of water-borne PGs alone (Sorensen et al. 1989). These endocrine and testicular responses to water-borne PGs appear not to be a result of direct pheromone exposure, but rather a result of increased male-female (and perhaps male-male) behavioral interactions which are induced by the postovulatory pheromone. For example, unlike 17,20B-P exposure, which induces endocrine responses in both grouped and isolated males (Stacey and Sorensen, 1986), water-borne PGs (or the odor of PG-injected females) increase GtH and milt levels in grouped males, which chase and "court" each other in response to exposure, but not in males tested in isolation (Sorensen et al. 1989). These findings indicate that the PG pheromone primarily stimulates male sexual arousal without directly affecting male GtH release (Sorensen et al. 1989), although in a normal social context (mixed sex group), PG pheromone exposure would be expected to result in concurrent behavioral and physiological responses. Since this interpretation of PG pheromone action is based on studies using only PG-injected females, the present study was conducted to determine whether stimuli from hCG-ovulated females would exert similar effects.

As expected, individual males allowed direct access to ovulated females had increased GtH levels within 1 h of spawning (Experiments 3 and 4). Furthermore, males permitted to spawn with PG-injected females had GtH increases which were identical to those of males which spawned with ovulated females (Experiment 4), supporting the proposal that ovulated females release a PG pheromone. However, individual males failed to exhibit a GtH response if they were separated from ovulated females by a partition that restricts physical contact but allows water exchange. The lack of response in these separated males may have been due to a lower pheromone concentration than that experienced by males allowed access to females. However, in a previous study, using

identical testing conditions (Stacey et al. 1989), separated and contact males showed similar GtH responses to 17,20B-P-releasing females. Thus, the present results provide further evidence that, although males become sexually aroused by the PG pheromone, they require behavioral interactions with PG-releasing females before GtH responses can be triggered.

These findings are consistent with other studies suggesting that the PG and 17,20B-P pheromone systems operate through different neuroendocrine mechanisms to regulate GtH release in males. For example, males exhibit equivalent endocrine responses to 17,20B-P at all times of the day, but exhibit a marked day-night difference in endocrine responsiveness to PG-releasing females (Chapter 6). As well, water-borne 17,20B-P and PGs are detected by different olfactory receptors (Sorensen et al. 1987; 1988) which may give rise to distinct neuroendocrine pathways. Although both pathways are known to course centrally through the medial olfactory tracts (MOT; Stacey and Kyle, 1983; Chapter 3), there is considerable evidence that the two pathways diverge at the level of the forebrain. Each of the two pheromone systems will be discussed separately below.

The GtH response to 17,20B-P in goldfish may be comparable to neuroendocrine reflexes in other vertebrates in that it occurs almost immediately following detection of the pheromone (Dulka et al. 1987a; Chapter 5). The response occurs in both grouped and isolated males (Experiments 2 and 5; Stacey and Sorensen, 1986), and is not influenced by social interactions with females (Stacey et al. 1989). Furthermore, GtH responses to supra-threshold concentrations of 17,20B-P do not appear to be dose dependent; relatively high concentrations (10^{-9} M) of water-borne 17,20B-P are no more effective at increasing GtH than threshold concentrations (10^{-11} M; Dulka et al. 1988; Chapter 4). Finally, although water-borne 17,20B-P stimulates GtH release within 15 min of exposure (Dulka et al. 1987), the magnitude of the response remains constant over time, even after continued exposure for up to 8-12 h (Chapter 5), and perhaps even after withdrawal of the stimulus (Chapter 2).

Taken together, these findings suggest that the preovulatory 17,20B-P pheromone activates an olfactory pathway that directly leads to reflex GtH responses in male goldfish (Chapter 5). For example, direct MOT projections to the antero-ventral preoptic area (POA)

have been identified in goldfish (Von Bartheld et al. 1984; Levine and Dethier, 1985; Springer, 1983). The POA contains both GnRH and dopamine (DA) immunoreactive cell bodies which project to the pituitary (Kah et al. 1986; Kah et al, 1987). As summarized in Figure 7.6, GtH secretion in goldfish is believed to be regulated by a combination of increased stimulation by GnRH and an abatement of DA inhibition (Peter et al. 1986). Based on the anatomical information available, it is proposed that reflex GtH responses to 17,20B-P involve the activation of a direct olfactory pathway to the POA (Fig. 7.6). It is not known however, whether 17,20B-P operates through GnRH, DA or the combined effects of both substances to cause increased GtH release in males.

In contrast to the situation for the 17,20B-P pheromone, GtH responses to PG-releasing females are clearly not reflexive in that they are dependent on the performance of socio-sexual behavior by the male. This suggests that GtH release in males is regulated, at least in part, by brain areas that control male socio-sexual behavior. Two areas of the ventral telencephalon, the *area ventralis telencephali pars supercommissuralis* (Vs) and the *area telencephali pars ventralis* (Vv), have been implicated in the control of male reproductive behavior in goldfish (Kyle et al. 1982; Kyle and Peter, 1892; Koyama et al. 1984). Both these areas receive olfactory projections from the MOT (Von Bartheld et al. 1984; Levine and Dethier, 1985; Springer, 1983). These projections appear to carry pheromonal information, since male sexual behavior is significantly reduced by lesions in Vv-Vs and/or the MOT (Kyle and Peter, 1982; Kyle et al. 1982; Koyama et al. 1984; Stacey and Kyle 1983). Because conspecific interactions appear necessary for PG-induced GtH increases (Sorensen et al. 1988), the Vv-Vs regions may stimulate the POA once the male's socio-sexual behavior is initiated. It is proposed that a functional Vv-Vs-preoptic connection (through the medial forebrain bundle) may serve as the anatomical substrate by which the PG pheromone can indirectly influence GtH release by activating male sexual behavior (Fig 7.6). Presumably, GtH release under these conditions would be regulated through GnRH and/or DA mechanisms similar to those regulating 17,20B-P pheromone system (see: Chapter 8).

Growth Hormone

Unlike male blood GtH, which increased rapidly and consistently in response to water-borne 17,20B-P or the odor of a preovulatory female, plasma levels of GH in males increased only following extended (8 h) exposure to a preovulatory female (Experiment 1). In more recent work (Chapter 5), chronic exposure to water-borne 17,20B-P increased male GH, but also only after 8 h of continuous exposure. However, this GH response to long term 17,20B-P exposure could not be repeated in a follow-up experiment (Chapter 5). Therefore, it seems likely that the preovulatory 17,20B-P pheromone increases GH indirectly. For example, since circulating gonadal steroids have been suggested to regulate GH release in other vertebrates (Dickerman et al. 1972), it is possible that 17,20B-P-induced GtH responses may indirectly influence GH release by first changing plasma levels of gonadal steroids. However, the effect of gonadal steroids on GH regulation in goldfish has not been examined in detail.

Changes in circulating GH concentrations were not observed in males exposed to postovulatory (PG-releasing) females. These results suggest that the PG pheromone has no direct effects on GH release, nor does the performance of spawning behavior influence GH release in males. However, if 17,20B-P-induced GtH increases eventually cause increased GH release through the actions of gonadal steroids (see above), then it would be expected that long term spawning stimuli would have a similar effect on male GH. This possibility, however, has not been examined in goldfish.

The lack of pronounced or consistent GH changes in males in this study is markedly different from the pattern of GH release in ovulating females. In female goldfish, GH increases in parallel with the GtH surge during spontaneous ovulation (Marchant, 1983). Increased levels of GH at ovulation suggest that this hormone plays an important role in female reproduction. In support, recent findings indicate that GH may potentiate the action of GtH on ovarian steroid production (G.J. Van Der Kraak and P.M. Rosenbloom, unpublished results). It is not known whether GH exerts a similar potentiating effect on

testicular steroid production. However, since males apparently lack pronounced GH changes during spawning, this phenomenon may be restricted to females. In the white sucker, *Catostomus commersoni*, the only other teleost in which both GH and GtH have been measured at spawning (Stacey et al. 1983), the patterns of GH and GtH release are similar to those described in goldfish (Marchant, 1983). For example, in female suckers, both GH and GtH levels are elevated during the ovulatory period and at spawning, whereas in naturally spawning male suckers, GtH levels are elevated but GH levels are not (Stacey et al. 1983).

The apparent absence of large GH increases in male goldfish (and perhaps white suckers) at spawning raises important questions regarding possible sex differences in the central regulation of GtH and GH release in this species. In females, GH release is closely associated with the occurrence of the ovulatory GtH surge, suggesting that the release of the two hormones is linked to common neural pathways or transmitter substances. In this regard, GnRH has been found to stimulate both GtH and GH release in female and male goldfish (Chang et al. 1982; Marchant, 1988). Although both pituitary hormones appear to share GnRH as a common releasing hormone, they are regulated by separate inhibitory mechanisms, DA inhibiting GtH and somatostatin (SRIF) inhibiting GH (Marchant et al. 1987). Thus, sex differences in GH and GtH levels at spawning could result if GnRH plays a dominant role in regulating GtH and GH release in females while the inhibitory systems predominate in males. In females, for example, the dual effect of GnRH on GtH and GH release could explain how these two hormones increase simultaneously in the blood during ovulation. If so, GnRH would not appear to play this role in males as GH changes do not accompany GtH increases. This lack of change in GH argues against a common GnRH stimulatory pathway in males, and indicates that abatement of DA inhibition could mediate male GtH responses to 17,20B-P and possibly to spawning females (see: Chapter 8). Finally, it should be noted that recrudescing females generally do not show GtH responses to 17,20B-P (Sorensen, unpublished results), whereas the pheromone increases the incidence of ovulation in sexually mature females (Sorensen and Stacey, 1987), presumably by evoking an ovulatory surge of GtH. This final point

strengthens the argument that males and females possess different neuroendocrine pathways to regulate GtH release, since females are as sensitive as males at detecting the pheromone at the level of the olfactory epithelium regardless of sexual condition (Sorensen et al 1987a). It also suggests that female sex pheromones operate through male-specific neuroendocrine pathways to caused increased GtH release under appropriate reproductive conditions.

In conclusion, the 17,20B-P and PG pheromones evidently act through different neuroendocrine mechanisms to regulate GtH release in male goldfish. Peak production and release of 17,20B-P by females occurs during the period of oocyte final maturation. The 17,20B-P pheromone appears to cause reflex GtH responses in males which may involve the activation of an olfactory-preoptic pathway. Elevated levels of GtH in males ultimately cause increased milt production by the time of ovulation (Dulka et al. 1987a). Production and release of PGs by females increases shortly after ovulation. Water-borne PGs primarily function to stimulate male sexual behavior (Sorensen et al. 1989). However, by stimulating brain regions that control male sexual activity, the PG pheromone may indirectly lead to increased GtH release in males. It seems likely that the function of elevated levels of GtH in spawning males is to enhance milt production for future spawning activity. This endocrine mechanism may work in concert with neurally mediated testicular and sperm duct contractions (Dulka and Demski, 1986) to increase the volume of milt that can be released during spawning (Kyle et al. 1985). In contrast to the close temporal relationship between GtH and GH release in ovulating female goldfish (Marchant, 1983), GtH release in pheromone-exposed males is not accompanied by GH release. The 17,20B-P and PG pheromones do not appear to stimulate GH directly, nor does the performance of spawning behavior influence GH release in males. Thus, both pheromones appear to operate through separate neuroendocrine pathways in males to specifically stimulate GtH release. Whether male and female goldfish use different neuroendocrine mechanisms to regulate GtH and GH release at spawning, and whether the male GtH responses to pheromones are mediated through changes in GnRH or DA activity, are two important, but unsolved problems (see: Chapter 8).

Figure 7.1. **A**: Serum 17,20B-P concentrations in saline-injected (SAL) and hCG-injected female goldfish which either failed to undergo oocyte final maturation (DUD), underwent oocyte maturation, but failed to ovulate (MAT), or ovulated (OV). Values are expressed as mean + sem. Sample sizes are indicated above each bar. **B** and **C**: Male GtH (**B**) and GH (**C**) responses following exposure to preovulatory saline-injected or hCG-injected females which underwent varying degrees of oocyte final maturation and/or ovulation (in **A**) by the end of the experiment. Sample sizes are indicated below each bar in **B**. Values are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001 vs SAL (ANOVA and Least Squares Means analysis).

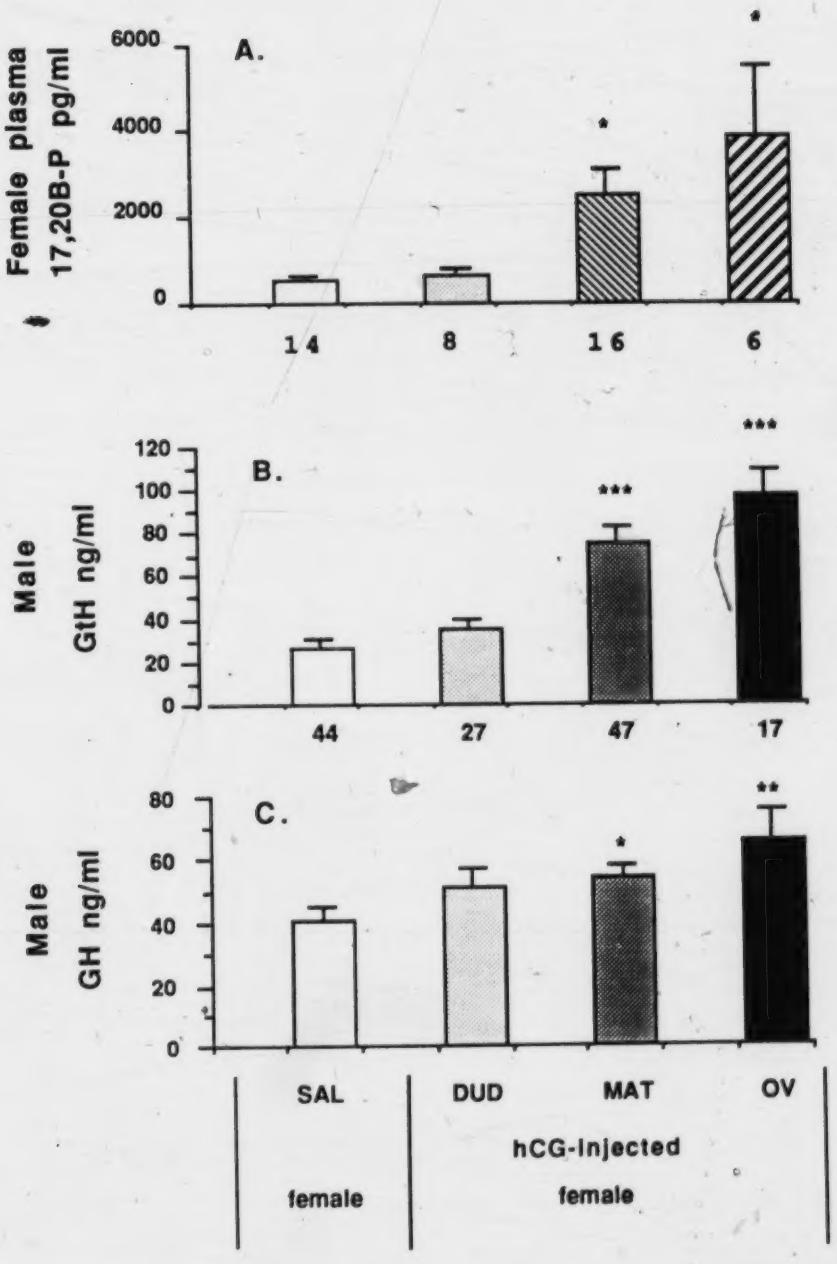

A.
Female plasma
17,20B-P pg/ml
6000
4000
2000
0
14
8
16
6
B.
Male
GtH ng/ml
120
100
80
60
40
20
0
44
27
47
17
***
***
C.
Male
GH ng/ml
80
60
40
20
0
SAL
DUD
MAT
OV
*
**
female
hCG-injected
female

Figure 7.2 Serum GtH (**A**) and GH (**B**) levels in groups of male goldfish following 1 h exposure to either 100 µl of ethanol or $5X10^{-10}$ M 17,20B-P. n=12 in all groups.
* $p<0.05$ vs EtOH.

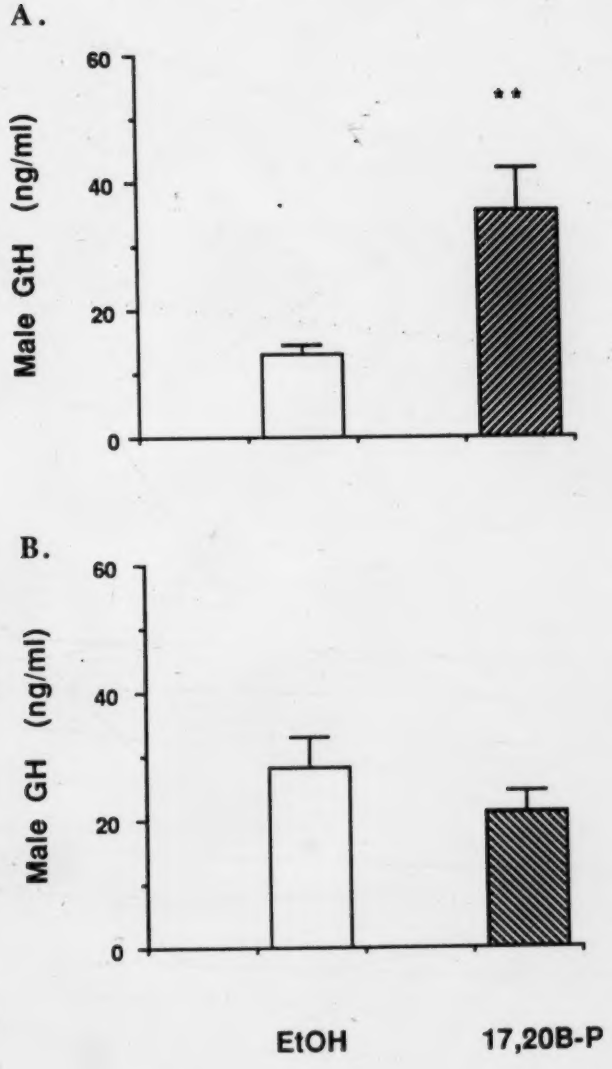

A.
Male GtH (ng/ml)
60
40
20
0
**
B.
Male GH (ng/ml)
60
40
20
0
EtOH
17,20B-P

Figure 7.3 Serum GtH (**A**) and GH (**B**) levels in male goldfish held individually in aquaria and exposed to nonovulatory female water (NONOV WATER), ovulatory female water (OV WATER), or allowed direct contact with an ovulatory female (OV CONT). Control males remained isolated (ISOL). Bars overlined by the same line are not significantly different ($p>0.05$, Student Newman Keuls multiple range test). n=12 in all groups.

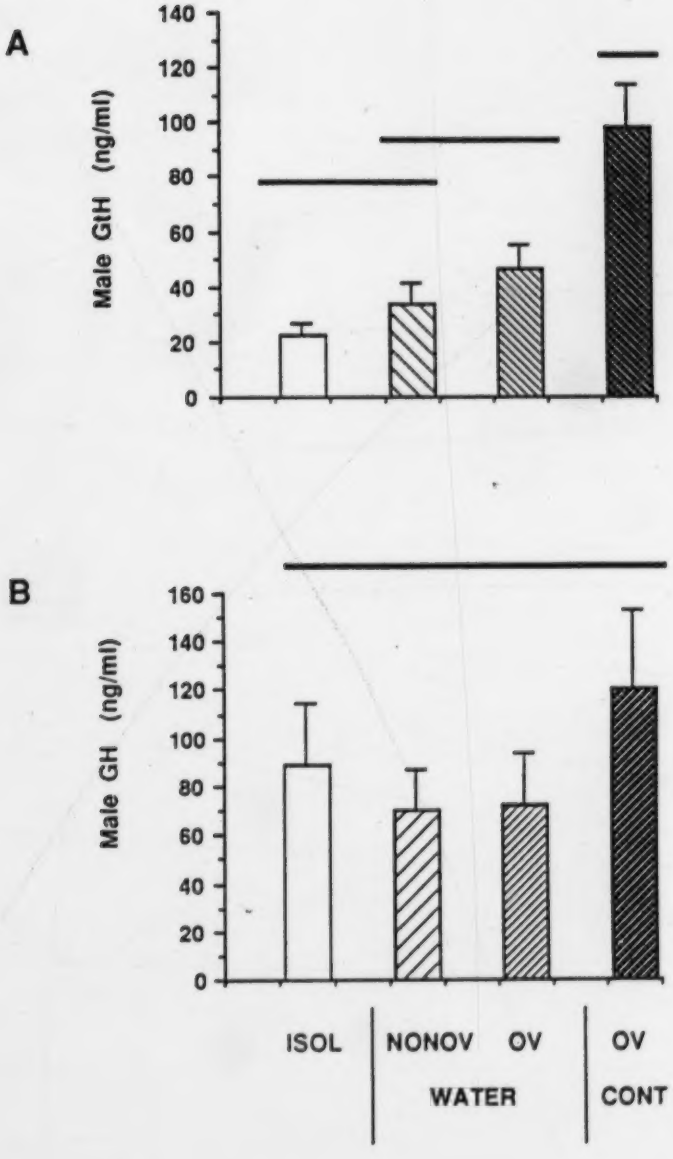

A
Male GtH (ng/ml)
140
120
100
80
60
40
20
0
B
Male GH (ng/ml)
160
140
120
100
80
60
40
20
0
ISOL
NONOV
OV
WATER
OV
CONT

Figure 7.4 Serum GtH (**A**) and GH (**B**) levels in male goldfish following exposure to either saline-injected (n=10), ovulated hCG-injected (n=14), or spawning PG-injected females (n=13). Groups overlined by the same line are not significantly different ($p>0.05$, Student Newman Keuls multiple range test).

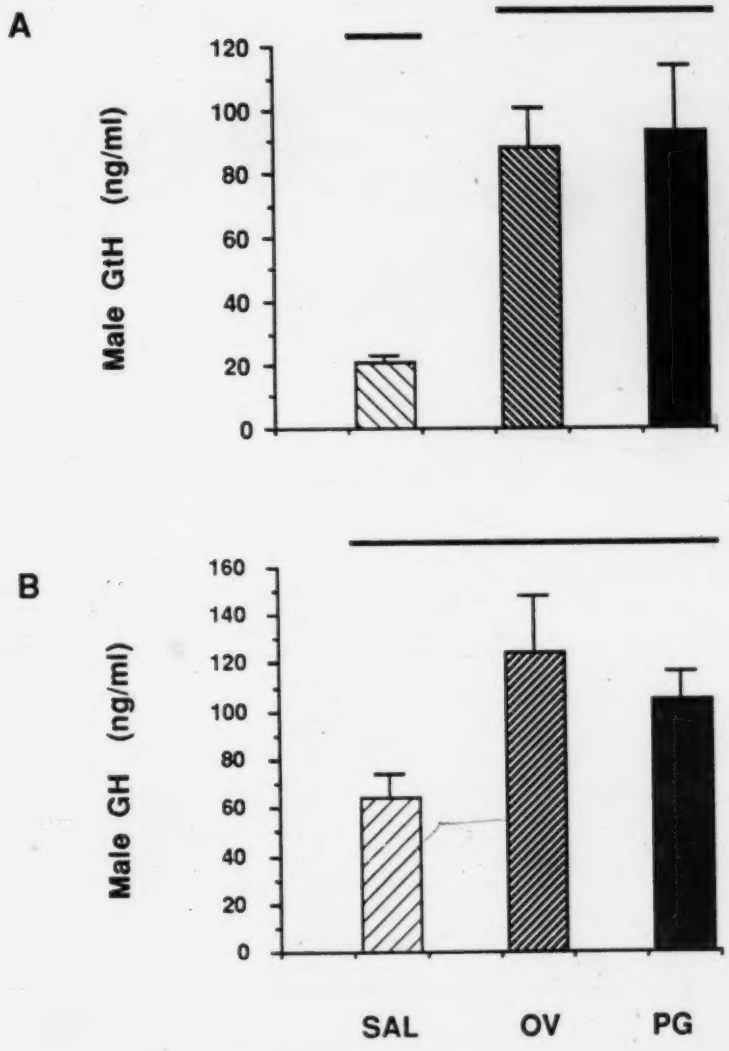

A
Male GtH (ng/ml)
120
100
80
60
40
20
0
B
Male GH (ng/ml)
160
140
120
100
80
60
40
20
0
SAL
OV
PG

Figure 7.5 Serum GtH (**A**) and GH (**B**) levels in isolated male goldfish exposed to one of four pheromone solutions at a concentration of 10^{-7} M (Con 1= ethanol control; PGs= mixture of $PGF_{2\alpha}$ and 15-$PGF_{2\alpha}$, P= 17,20B-P; PG fem= PG-injected female water), or one of four behavioral stimuli (Con 2= isolated males, no treatment; Sal fem= saline-injected female; PG fem= PG-injected female; Fd= food). n=13 in all groups. * $p<0.05$, ** $p<0.01$ compared to the appropriate control group (CON1 or CON2).

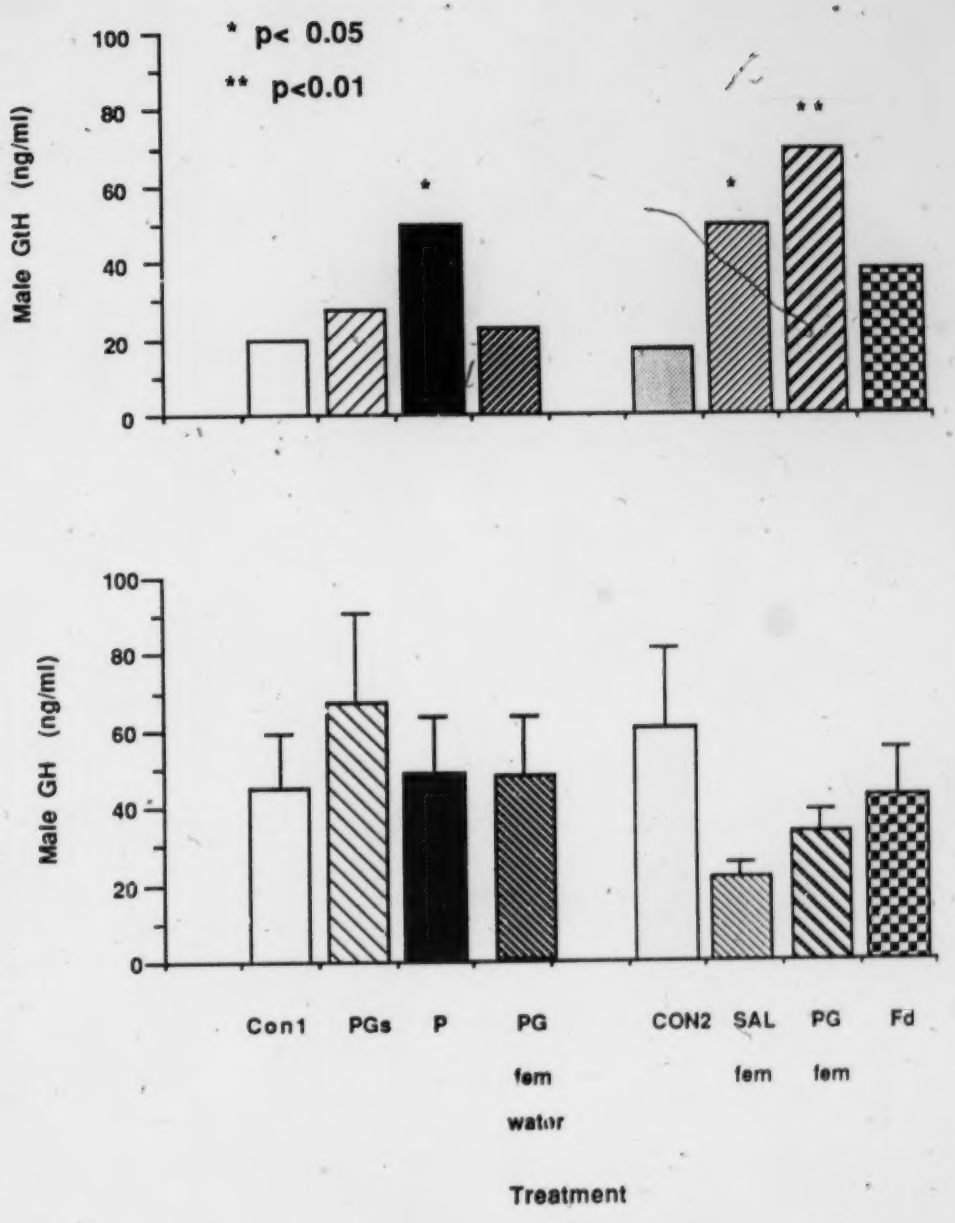
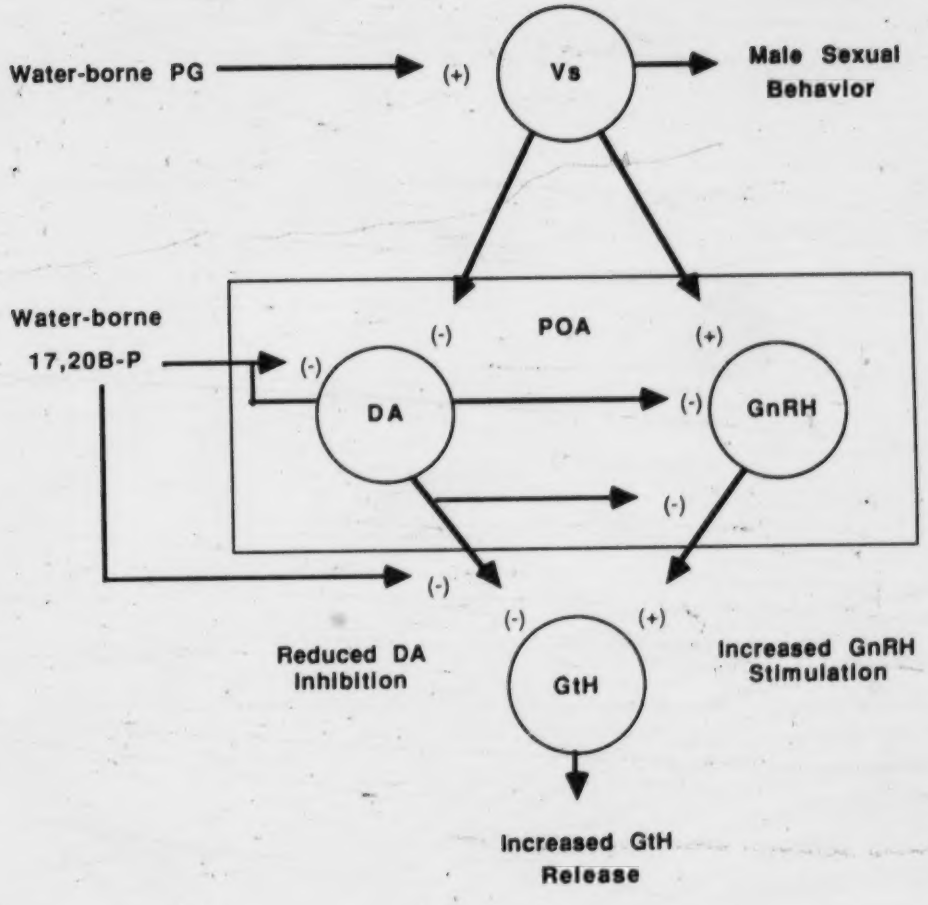

* p< 0.05
** p<0.01
Male GtH (ng/ml)
100
80
60
40
20
0
Male GH (ng/ml)
Con1
PGs
P
PG fem water
CON2
SAL fem
PG fem
Fd
Treatment

Figure 7.6. Proposed model of the neuroendocrine regulation of pheromone-induced GtH release in male goldfish. Both the 17,20B-P and PG pheromone systems are considered. It is proposed that 17,20B-P triggers reflex GtH responses through the activation of a direct projection to the preoptic area (POA). Water-borne 17,20B-P may influence GtH release by causing reduced DA inhibition and/or increased GnRH stimulation at the level of the pituitary. Although the PG pheromone does not appear to affect GtH directly, it functions primarily to stimulate male sexual behavior, presumably by activating behavioral centers in the *area ventralis telencephali pars supercommissuralis* (Vs). However, the PG pheromone may indirectly result in increased GtH release by connections between Vs and the POA. (-) and (+) indicate inhibition and stimulation, respectively.


Water-borne PG
(+)
Vs
Male Sexual Behavior
Water-borne 17,20B-P
POA
(-)
(+)
(-)
DA
(-)
GnRH
(-)
(-)
(-)
(-)
(+)
Reduced DA Inhibition
GtH
Increased GnRH Stimulation
Increased GtH Release

7.5 LITERATURE CITED

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8. INVOLVEMENT OF DOPAMINE IN THE CENTRAL CONTROL OF GONADOTROPIN SECRETION IN RESPONSE TO A PREOVULATORY FEMALE SEX PHEROMONE IN MALE GOLDFISH

8.1 INTRODUCTION

Shortly before ovulation, female goldfish release the gonadal steroid, 17α,20B-dihydroxy-4-pregnen-3-one (17,20B-P), which acts as a potent sex pheromone (Dulka et al., 1987a; Stacey et al., 1989). Water-borne 17,20B-P is readily detected by males (Sorensen et al., 1987, 1989) and induces a rapid increase in blood gonadotropin (GtH) which subsequently increases milt (sperm and seminal fluid) production by the time females ovulate (Dulka et al., 1987a; Stacey et al., 1989). Both the temporal pattern of 17,20B-P release from preovulatory females (Stacey et al., 1989), and the latency of the males endocrine responses to water-borne 17,20B-P (Chapter 6) suggest that this pheromone assists in synchronizing reproduction in goldfish (Dulka et al., 1987a).

The neuroendocrine mechanisms by which water-borne 17,20B-P elevates male GtH are poorly understood. Specific olfactory receptors which detect 17,20B-P (Sorensen et al., 1987, 1989) appear to give rise to a central pathway which courses through the medial olfactory tracts (Stacey and Sorensen, 1986; Chapter 3) to innervate various regions of the forebrain. At least some of these projections terminate in the anteroventral preoptic area (POA; von Bartheld et al., 1984; Levine and Dethier, 1985; Kyle et al., 1987), a region previously implicated in the control of GtH secretion in goldfish and other teleosts (Peter et al., 1986).

In goldfish, GtH release is under the stimulatory influence of gonadotropin-releasing-hormone (GnRH) and the inhibitory control of dopamine (DA) (Peter et al., 1986). In addition, immunoreactive cell bodies for both GnRH and DA have been localized in the anteroventral POA in goldfish (Kah et al., 1984a,b, 1986, 1987). Unlike tetrapods, which

possess a well defined median eminence and a hypothalamic portal system (see Peter et al., 1986), the preoptic GnRH and DA cell bodies in teleosts project directly to the pituitary gland (PIT) to innervate the gonadotropes (Kah et al., 1986, 1987). It is through this projection that both GnRH and DA are believed to regulate GtH release in goldfish (see Peter et al., 1986).

Chang et al. (1983) first demonstrated the involvement of DA in the neuroendocrine regulation of GtH release in goldfish. For example, intraperitoneal injections of 6-hydroxydopamine and reserpine, which cause a general depletion of DA in nerve terminals, were found to increase circulating GtH levels in female goldfish (Chang et al.,, 1983). Similar effects on GtH release were observed following injection of various DA receptor antagonists such as pimozide, metoclopramide and domperidone (Chang and Peter, 1983a,b, Chang et al., 1984; Sokolowska et al., 1984; Omelyjaniuk et al., 1987). In addition, injections of drugs such as o -methyl-para-tyrosine and carbidopa, which block L-dopa and DA synthesis, respectively, elevate, whereas injections of DA and DA agonists suppress, GtH release in goldfish (Chang et al., 1983). Subsequent *in vivo* and *in vitro* studies have demonstrated that DA acts directly on the gonadotropes to inhibit GtH release (see Peter et al., 1986). However, DA also inhibits GnRH-stimulated GtH release in goldfish (Chang and Peter, 1983a; Chang et al., 1984b) presumably by inhibiting spontaneous GtH release and suppressing the stimulatory actions of GnRH (Peter et al., 1986).

As in other vertebrates, the goldfish brain contains multiple forms of GnRH (Sherwood, 1986), two of which have been identified (Sherwood et al., 1983; Yu et al., 1988). The major form of goldfish GnRH has been shown to be chromatographically and immunologically identical to [Trp^7, Leu^8]-GnRH (salmon GnRH or sGnRH), whereas the second form is similar, if not identical, to [His^5, Trp^7, Try^8]-GnRH (chicken GnRH-II or cGnRH-II; Yu et al., 1988). Although the proportion of immunoreactive cGnRH-II to immunoreactive sGnRH is higher in the caudal brain compared to the rostral brain, both of these compounds are widely distributed in the goldfish brain (Yu et al., 1988). However, at present, it is unclear which of these forms of GnRH, if not both, participates in the

neuroendocrine regulation of GtH secretion in goldfish.

Structure-activity relationships of various forms of GnRH on GtH release in goldfish have been recently examined both *in vivo* and *in vitro* (Peter et al., 1985, 1987). These studies have shown that many forms of GnRH (mammalian, chicken and salmon) stimulate GtH release *in vivo*, but generally only when injected in combination with DA antagonists (Peter et al 1985, 1986). Thus, although native forms of GnRH (sGnRH and cGnRH-II) in goldfish are capable of stimulating GtH release, the stimulatory actions of these compounds appear to be directly influenced by the degree in DA inhibition present at the PIT.

The present study examined whether the male GtH response to water-borne 17,20B-P is mediated through the actions of GnRH and/or DA in goldfish. Possible changes in brain and PIT levels of DA, as well as other catecholamines, were monitored using high performance liquid chromatography with electrochemical detection (HPLC/ED). The possibility that water-borne 17,20B-P may induce changes in the rate of DA turnover in the PIT was also examined. We also tested whether DA synthesis blockers would affect the male GtH response to water-borne 17,20B-P. In addition, possible pheromone-induced changes in brain and PIT levels of sGnRH and/or cGnRH-II were monitored using recently developed radioimmunoassay systems for each form of GnRH.

8.2 MATERIALS AND METHODS

Animals

Goldfish of the common or comet variety (20-80 g) were purchased from Ozark Fisheries Co. Stoutland, Missouri. Sexually mature males having well developed pectoral tubercles (pearl organs) and expressible milt were maintained in 1000 L flow-through stock aquaria at 15-20^0 C under a 16L:8D photoperiod (lights on at 08:00 h). Fish were fed Ewos trout pellets and Nutrafin flaked food *ad libitum* at least once a day.

General Methods

Except in Experiments 4 and 5, in which fish were first injected with a DA synthesis blocker (see below), the following procedures were common to all experiments. Groups of males were transferred from stock aquaria to 65 l flow-through test aquaria (3 fish per aquarium; 20° C; 16L:8D) at least two days before each experiment was conducted. All test aquaria contained gravel substrate, aeration stones and were covered by opaque barriers to minimize external disturbances. On the day of the experiment, the water flow to the appropriate aquaria was shut off and either 100 µl of ethanol (Control) or 10 µg 17,20B-P (Sigma) in 100 µl ethanol was injected directly into the aeration bubbles to ensure rapid dilution of the steroid. Although the resulting 17,20B-P water concentrations were not measured, the amount added to each aquarium should have created a final water concentration of approximately $5X10^{-10}$ M. Following various durations of exposure, the fish were removed, anesthetized in a 0.05% solution of 2-phenoxyethanol (Syndel) and blood sampled through the caudal vasculature for GtH determination. 2-phenoxyethanol has no effect on brain amines when used as an anesthetic (Sloley et al., 1986).

Immediately after blood sampling, the brains of all fish were removed and dissected into appropriate regions as summarized in Figure 8.0. The collected brain areas included the olfactory bulbs (OB), telencephalon (TEL), hypothalamus (HYP) and pituitary (PIT); however, not all brain areas were collected in every experiment. Upon dissection, each brain region was either immediately immersed in 2.0 ml of ice cold 2 M acetic acid for extraction of brain GnRH (see below), or placed in a pre-weighed polypropylene micro-centrifuge tube before being frozen on dry ice. These tubes were re-weighed before adding 30-150 µl of 0.2 N perchloric acid to extract biogenic amines (Sloley et al., 1986). The perchloric acid solution contained 50 ng/ml isoproterenol as an internal standard. Extraction of amines was facilitated by homogenizing each sample for 10 s using a micro-ultrasonic cell disrupter (Kontes). The extracted samples were centrifuged (14,000 g) at room temperature for 15 min and a 20 µl aliquot of supernatant applied directly to a high-pressure liquid chromatography (HPLC) column.

Concentrations of brain amines were determined by HPLC/ED modified from Sloley et al. (1986). Identification of compounds was done by co-chromatography with known standards and by their electrochemical characteristics. The glassy carbon detector (BAS, West Lafayette, IN, USA) was operated at 0.65 V (versus Ag/AgCl) to determine noradrenaline (NA), dopamine (DA) and its metabolite, dihydroxyphenylacetic acid (DOPAC), and serotonin (5HT) and its metabolite, 5-hydroxyindoleacetic acid (5HIAA), from paired OBs and individual pieces of TEL, HYP, and PIT. Separation of these compounds was achieved using a 250 X 4.6 mm (inside diameter) column packed with 5 um spherical particles (Supelco, Bellefonte, PA., USA) and protected by a Brownlee RP-18 15 X 3.2 mm guard column (Brownlee, Santa Clara, Ca., USA). The mobile phase consisted of 75 mM NaH_2PO_4, 1 mM octylsodium sulphate, 0.05 M EDTA and 13% acetonitrile. The pH of the final mobile phase was adjusted to 2.75 with concentrated H_3PO_4 and pumped by a Waters M-590 solvent delivery system (Waters, Mississauga, Ontario) at a flow rate of 1.0 ml/min. Aliquots (20 µl) were applied to the column by a Rheodyne injector.

Extraction and measurement (RIA) of immunoreactive sGnRH in brain was conducted according to methods described by Yu et al. (1988). The sGnRH antiserum exhibits approximately 30% cross-reactivity with cGnRH-II (Yu et al., 1987; personal communication). Brain levels of cGnRH-II in the same samples were determined by (RIA) according to methods described by Huang et al. (in preparation). The cGnRH-II antiserum exhibits less than 10% cross-reactivity with sGnRH (Y.P. Huang, personal communication).

Blood samples were centrifuged (14,000 g) at 4° C for 15 min to obtain serum which was then stored frozen (-30° C) until assayed. Serum concentrations of GtH were determined by radioimmunoassay (RIA) as described previously (Peter et al., 1984).

Experiment 1

This experiment examined whether exposure to water-borne 17,20B-P causes changes in biogenic amines in various brain regions of male goldfish. Groups (n=6) of

males were exposed to ethanol or 17,20B-P for either 15 min, 1 h, or 4 h and then blood sampled for GtH determination. The brains of all fish were dissected for HPLC determination of amine levels in OB, TEL, HYP and PIT. Brain GnRH levels were not measured in this experiment.

Experiment 2

This experiment examined whether water-borne 17,20B-P causes short-term changes in DA turnover in the PIT and/or HYP of male goldfish. Possible changes in DA turnover were assessed by measuring tissue concentrations of both DA and DOPAC and by comparing the ratio of DOPAC to DA in tissues obtained from ethanol and 17,20B-P-exposed males. Since PIT concentrations of DOPAC were low and difficult to measure in the relatively small (20-30 g) fish used in Experiment 1, larger (60-80 g) sexually mature males were used in this experiment in order to maximize the chances of accurately measuring DOPAC in the PIT. Groups (n=18 per group, 3 fish/aquarium) of males were exposed to either ethanol for 20 min or 17,20B-P for 20 min or 45 min. Blood samples for GtH determination were taken at each sample time. Immediately after blood sampling, the HYP and PIT were removed and later processed for measurement of DA and DOPAC by HPLC/ED.

Experiment 3

This experiment was conducted to verify whether water-borne 17,20B-P causes a reduction in DA turnover in the PIT of male goldfish. The males (60-80 g) used in this experiment were from the same stock of fish used in Experiment 2. Paired groups of males were exposed to either ethanol or 17,20B-P for 45 min (n=15) or 2 h (n=9). Blood and tissue (HYP and PIT) samples also were taken from a fifth group of fish which was not exposed to any odor. Measurement of HYP and PIT concentrations of DA and DOPAC was conducted exactly as described for Experiment 2.

Experiment 4

This experiment was conducted to determine if DA synthesis blockers affect the GtH response to water-borne 17,20B-P and whether such possible affects can be attributed to changes in HYP and/or PIT DA content. Two DA synthesis blockers, carbidopa (CARB) and α-methyl-para-tyrosine (MPT), which have been shown to alter basal GtH in goldfish (Chang et al., 1983), were used under two different testing conditions.

In the first condition, one group of males (n=8) was injected (ip) with a saline suspension of CARB (50 µg/g Bd.Wt.) while a second group was injected with MPT (300 µg/g Bd.Wt.). A third group of males (Controls) was injected with saline vehicle (5.0 µl/g Bd.Wt.) alone. All injections were completed one hour before the fish were exposed to ethanol (Control) or water-borne 17,20B-P. One half (n=4, 2 fish/aquarium) of the fish in each treatment group was exposed to ethanol while the other half was exposed to 17,20B-P. All exposures lasted 12 h (overnight), after which the fish were blood sampled for GtH determination. Immediately after blood sampling, the brains of all fish were removed and dissected to collect samples of HYP and PIT. Amine levels in HYP and PIT were determined by HPLC/ED.

The second testing condition was identical to the first, except that the exposures were delayed until 13 h after drug treatment and the exposures lasted for only 4 h. In this way, fish tested under the second condition were treated with the drugs for a total of 17 h and were exposed to ethanol or 17,20B-P over only the latter 4 h of drug treatment. In contrast, the fish tested under the first condition were exposed to ethanol or 17,20B-P for 12 of the 13 h that they were treated with the drugs. Brain levels of GnRH were not measured under either condition.

Experiment 5

This experiment was conducted to determine if CARB potentiates the GtH response to water-borne 17,20B-P and whether such an effect can be attributed to changes in HYP or PIT content of DA and/or GnRH. Injections (saline and CARB) and exposures (ethanol

and 17,20B-P) were as described for the first testing condition in experiment 2 (13 h drug treatment; 12 h pheromone exposure). However, in each treatment group, one half (n=12) of the tissue samples was processed for measurement of biogenic amines while the other half was processed for measurement of sGnRH and cGnRH-II.

Statistical Analysis

All data were tested for homogeneity of variances using Bartlett's test (Biostat 1, Sigma Soft, Placentia, Ca. USA). When appropriate, data were normalized using either log10 or square root transformations. In Experiments 1, 4, and 5, serum GtH and brain levels of catecholamines and/or GnRH were analyzed with 2-way ANOVA and Least Squares Means (LSM) follow-up tests using a SAS computer package (SAS Institute, Cary, NC. USA). In Experiments 2 and 3, serum GtH and brain levels of catecholamines and GnRH were analyzed with one-way ANOVA and LSM follow-up tests.

8.3 RESULTS

Experiment 1

Serum GtH levels in ethanol-exposed males remained low and were not significantly different from each other (Fig. 8.1A). In contrast, males exposed to water-borne 17,20B-P had significantly ($p<0.05$) higher GtH levels than ethanol-exposed controls following 15 min, 1 h, and 4 h of exposure to the pheromone (Fig. 8.1A).

The OBs of control animals had relatively high levels of NA, intermediate levels of DA, lower levels of 5HT, and low to non-detectable levels of DOPAC and 5HIAA (Fig. 8.1B). All amine levels were similar between ethanol- and 17,20B-P-exposed males at each sample time (Fig. 8.1B).

In general, the TEL and HYP of control animals had relatively high levels of NA, intermediate levels of 5HT, lower levels of DA, and low to non-detectable levels of DOPAC and 5HIAA (Figs. 8.1C 8.1D). Water-borne 17,20B-P had no effect on amine

levels in TEL and PIT at all exposure times (Figs. 8.1C, 8.1D).

PIT levels of NA, 5HT, and 5HIAA were very low and therefore not determined in the control and experimental groups of males. However, control males had high PIT concentrations of DA and low to non-detectable levels of DOPAC (Fig. 8.1E). Exposure to water-borne 17,20B-P caused a slight increase in PIT concentrations of DA and DOPAC over 4 h of exposure. However, PIT concentrations of DA and DOPAC in these animals were statistically similar to those of ethanol-exposed males at each sample time (Fig. 8.1E).

Experiment 2

Serum GtH levels in males exposed to water-borne 17,20B-P for 20 min and 45 min were significantly ($p<0.01$) higher than those of males exposed to ethanol for 20 min (Fig. 8.2A). In the same fish, DA levels in the PIT were similar among all groups, regardless of whether the animals were exposed to ethanol or 17,20B-P (Fig. 8.2B). However, those males exposed to 17,20B-P for 45 min had significantly ($p<0.01$) lower levels of DOPAC in the PIT than did males exposed to ethanol or 17,20B-P for 20 min (Fig. 8.2C). In addition, the ratio of DOPAC to DA content in the PITs of both 17,20B-P-exposed groups was significantly ($p<0.05$) lower than that of the ethanol-exposed group (Fig. 8.2D), suggesting that water-borne 17,20B-P causes a reduction of DA turn-over in the PIT.

Experiment 3

Serum concentrations of GtH in the non-exposed and the ethanol-exposed groups were similar and not significantly different from each other (Fig. 8.3A). In contrast, males exposed to 17,20B-P had significantly ($p<0.01$) higher GtH levels than those of males exposed to ethanol for 20 min and 45 min (Fig 8.3A). As in Experiment 2, DA levels in the PIT were similar in all groups of fish (Fig. 8.3B). In contrast, PIT concentrations of DOPAC in 17,20B-P-exposed males were significantly ($p<0.01$) lower than those of ethanol-exposed males at 45 min, but not at 2 h (Fig. 8.3C). However, as in Experiment 2, the ratio of DOPAC to DA content in the PIT was significantly ($p<0.05$) lower in 17,20B-P-exposed males compared to ethanol-exposed fish at each sample time (Fig. 8.3D).

Experiment 4

The GtH levels of saline and drug-treated (CARB and MPT) males examined under both testing conditions are summarized in figures 8.4A and 8.4B. In the first testing condition (13 h drug treatment; 12 h pheromone exposure), statistical analysis (2-way ANOVA) of the GtH data indicated a significant effect of drug ($p<0.001$), odor ($p<0.001$) and a drug-odor interaction ($p<0.01$). However, statistical follow-up tests (Duncan's multiple range tests, Least Squares Means analysis) failed to indicate differences between some of the groups. For example, compared to ethanol-exposed controls, a significant effect of 17,20B-P exposure was not observed in saline-injected males. Based on the results of experiment 1, it is likely that water-borne 17,20B-P stimulated GtH release in this experiment. In the first testing condition (13 h drug treatment; 12 h pheromone exposure), CARB alone caused an increase in male GtH release similar to that caused by water-borne 17,20B-P alone (Fig. 8.4A). However, CARB-injected 17,20B-P-exposed males had significantly ($p<0.05$) greater GtH responses than did saline-injected 17,20B-P-exposed males. Since the combined effects of CARB and 17,20B-P-exposure appear to be more than additive (Fig. 8.4A), the results suggest that this form of manipulation leads to a potentiation of the GtH responses to water-borne 17,20B-P. In contrast, although GtH levels in MPT-injected ethanol-exposed males were significantly ($p<0.05$) higher than saline-injected and CARB-injected fish exposed to ethanol, and higher than saline-injected fish exposed to 17,20B-P, they were similar to CARB-injected and MPT-injected fish exposed to water-borne 17,20B-P (Fig 8.4A).

The above GtH responses appear to reflect corresponding drug-induced changes in DA content in the HYP and PIT. For example, compared to saline-injected males, CARB, which presumably does not cross the blood brain barrier (Gilman et al., 1985), caused a significant ($p<0.05$) decrease in DA content in the PIT, but not the HYP (Figs. 8.4C, 8.4D). In contrast, MPT, which is known to cross the blood brain barrier in mammals and other vertebrates (Gilman et al., 1985), caused a dramatic reduction of DA content in both the HYP and PIT (Figs. 8.4C, 8.4D). Although these results may explain the GtH

responses observed in ethanol-exposed males (Fig. 8.4A), the GtH responses to water-borne 17,20B-P are more difficult to explain since HYP and PIT levels of DA do not appear to be affected by 17,20B-P-exposure (Figs. 8.4C, 8.4D).

A possible potentiating effect of CARB and/or MPT on the 17,20B-P-induced GtH response was not observed in fish examined under the second testing condition (17 h drug treatment; 4 h pheromone exposure) even though central effects were still evident. For example, CARB-injected males had significantly ($p<0.05$) lower PIT levels of DA than saline-injected controls (Fig. 8.4E). Exposure to water-borne 17,20B-P had no effect on DA levels in CARB-injected fish (Figs. 8.4E, 8.4F). In addition, MPT reduced DA levels in both the HYP and PIT, regardless of whether the animals were exposed to ethanol or 17,20B-P (Fig. 8.4F). These results suggest that the central effects of the drugs had not worn off by the time the fish were sampled under the second testing condition. Instead, it appears that exposure times of greater than 4 h are required for the combination of CARB and water-borne 17,20B-P to cause greater GtH responses than water-borne 17,20B-P alone.

Experiment 5

The GtH data from this experiment are presented three ways. To allow comparisons between changes in GtH release and possible changes in brain levels of DA and/or GnRH(s), the GtH data are presented separately for the groups in which these compounds were measured. Those fish which were processed for measurement of HYP and PIT levels of DA will be referred to as the DA-fish, while those fish processed for sGnRH and cGnRH-II will be referred to as the GnRH-fish. In the third case, the GtH data from both groups of fish were combined since the animals were treated identically, regardless of which brain compounds were measured at the end of the experiment. The combined GtH data will therefore be referred to as that of the combined-fish.

In the DA-fish, exposure of saline-injected males to water-borne 17,20B-P caused a significant ($p<0.01$) increase in GtH compared to saline-injected fish exposed to ethanol (Fig. 8.5A). As in Experiment 2 (see above), CARB alone caused an increase in GtH

release similar to that caused by water-borne 17,20B-P alone (Fig. 8.5A) and significantly ($p<0.05$) higher than those of saline-injected ethanol-exposed males (Fig 8.5A). CARB-injected males exposed to water-borne 17,20B-P had significantly ($p<0.05$) higher GtH levels than CARB-injected males exposed to ethanol (Fig. 8.5A). However, unlike the previous experiment, there was no significant difference in GtH responses between saline-injected and CARB-injected fish exposed to water-borne 17,20B-P (Fig 8.5A).

In the GnRH-fish, exposure of saline-injected males to water-borne 17,20B-P caused a moderate, but significant ($p<0.01$), increase in GtH compared to saline-injected ethanol-exposed controls (Fig. 8.5B). As was observed in the DA-fish, CARB alone significantly ($p<0.01$) increased GtH to levels similar to those observed in fish exposed to water-borne 17,20B-P alone (Fig. 8.5B). However, unlike the DA-fish, the combined effects of CARB and 17,20B-P-exposure caused significantly ($p<0.01$) greater GtH responses than exposure to 17,20B-P alone (Fig. 8.5B).

The GtH data of the combined-fish are similar to those of the GnRH-fish (Fig. 8.5C). Thus, the overall effects of CARB and 17,20B-P-exposure appear to cause significantly ($p<0.05$) greater GtH responses than exposure to water-borne 17,20B-P alone (Fig. 8.5C).

In the DA-fish, the HYP of saline-injected animals had relatively high levels of NA, and slightly lower, but equivalent, levels of DA and 5HT (Figs. 8.5D-F). HYP levels of these amines were not effected by CARB. In addition, exposure to water-borne 17,20B-P failed to alter NA, DA or 5HT levels in the HYP, regardless of whether the animals were injected with saline or CARB (Figs. 8.5D-F).

The PIT of saline-injected animals had relatively high levels of DA, lower, but intermediate levels of NA, and low, but detectable, levels of 5HT (Figs. 8.5G-I). CARB alone caused a significant ($p<0.05$) reduction of DA content in the PIT without affecting levels of NA and 5HT (Figs. 8.5G-I). This effect was not observed in the HYP (Fig. 8.5F). Exposure to water-borne 17,20B-P had no effect on any amine levels, regardless of whether the fish were injected with saline or CARB (Figs. 8.5G-I).

In the GnRH-fish, CARB had no effect on sGnRH levels in the brain or PIT (Figs.

8.5J-L). Although CARB-injected 17,20B-P-exposed males appeared to have higher levels of sGnRH in the TEL and PIT, these levels were not significantly different (Figs. 8.5J-L). In general, 17,20B-P exposure had no effect on brain or PIT levels of sGnRH.

In the same animals, cGnRH-II levels in the TEL were similar in all treatment groups (Fig. 8.5M). In contrast, statistical analysis (2-way ANOVA) of cGnRH-II levels in the HYP indicated a significant effect of odor (ethanol vs. 17,20B-P; $p<0.05$), but no effect of drug or a drug-odor interaction. For example, in CARB-injected fish, those males exposed to water-borne 17,20B-P had significantly ($p<0.05$) higher levels of cGnRH-II in the HYP than did males exposed to ethanol (Fig. 8.5N). However, cGnRH-II levels in the CARB-injected 17,20B-P-exposed group were no different than those of males injected with saline and exposed to water-borne 17,20B-P (Fig. 8.5N). In general, CARB alone had no effect on cGnRH-II levels in the brain or PIT (Figs. 8.5M-O).

Statistical analysis (2-way ANOVA) of cGnRH-II levels in the PIT indicated a significant drug-odor interaction, but no effect of drug or odor alone. In the PIT, cGnRH-II levels were similar in ethanol and 17,20B-P-exposed males injected with saline, and in ethanol-exposed males injected with CARB (Fig. 8.5O). However, cGnRH-II levels in CARB-injected 17,20B-P-exposed males were significantly ($p<0.05$) higher than CARB-injected ethanol-exposed and saline-injected 17,20B-P-exposed males (Fig. 8.3O). The results suggest that the combined effects of CARB and water-borne 17,20B-P cause increased accumulation of cGnRH-II in the PIT. Interestingly, this effect is similar to that observed on GtH release in the same animals (Fig. 8.5B).

8.4 DISCUSSION

The present study is the first to examine the central control of an endocrine response to an identified sex pheromone in a teleost. In goldfish, 17,20B-P is released by preovulatory females as a sex pheromone which stimulates rapid increases in serum GtH

and milt production in males (Dulka et al., 1987a; Stacey et al., 1989; Chapters 2-7). GtH release in goldfish is believed to be regulated by the brain through the stimulatory actions of GnRH and the inhibitory actions of DA (Peter et al., 1986). However, although changes in brain GnRH levels have been correlated with patterns of GtH release during spawning in some teleosts (see below), there have been no reports on functional changes in brain levels of DA or how such changes influence GtH release. In this regard, the present study used a variety of techniques to examine whether the male GtH response to water-borne 17,20B-P is mediated through the actions of GnRH and/or DA in goldfish.

As in previous studies (Chapters 2-7), exposure to water-borne 17,20B-P consistently elevated GtH levels in male goldfish. A DA-mediated mechanism appears to be involved in controlling male GtH responses to the pheromone since 17,20B-P caused a clear and consistent reduction of DA turnover in the pituitary (Experiments 4 and 5).

However, initial attempts at measuring changes in brain and PIT levels of DA failed to indicate a clear relationship between the possible involvement of this compound and GtH release. In other words, the measurement of DA alone, either in the brain or PIT, did not provide an accurate indication of 17,20B-P-induced changes in DA activity. For example, pheromone-induced changes in DA levels were not observed in any brain area examined in the present study. These results appear similar to those of mammalian studies in which resting DA concentrations remain remarkably constant in a variety of tissues despite very different rates of neuronal depolarization and transmitter release (see review: Barraclough and Wise, 1982). However, in the present study, it is possible that the lack of differences in amine levels between treatment groups is due to the relatively large areas of brain that were collected for assay. For example, a more focused study of changes in amine levels in specific brain nuclei may reveal differences between ethanol and 17,20B-P-exposed groups.

In contrast, a clear pheromone-induced reduction in DA activity was demonstrated only when it was possible to measure DOPAC along with DA. Alterations in the rate of DA turnover, as determined by measuring the conversion of DA to DOPAC, serves as a reliable indicator of DA release in mammals (Roffer-Tarlov et al., 1971). Furthermore, in

mammals, increased turnover rates or rates of synthesis have been equated with increased transmitter release, whereas decreased turnover rates are associated with reduced transmitter release and decreased neuronal activity (see review: Barraclough and Wise, 1982). In this regard, changes in the ratio of DOPAC to DA content measured in the PIT provided the most accurate means of assessing the effects of water-borne 17,20B-P on DA turnover and GtH release in male goldfish. Since the reduction of DA turnover in the PIT is inversely correlated with periods of increased GtH release, the present results suggest that water-borne 17,20B-P causes an abatement of DA release to the PIT.

An important point to note is that DOPAC concentrations in the PIT were measured with accuracy only in relatively large (60-80 g) goldfish. The PITs of smaller (20-30 g) fish do not appear to contain high enough amounts of DOPAC to allow precise measurement by HPLC/ED. This may be related, in part, to rapid clearance of DOPAC from the PIT by the circulation. However, based on measurements of whole PITs, it is reasonable to assume that larger fish contain more DOPAC than smaller fish.

Pheromonal 17,20B-P input to the forebrain may affect GtH release by altering the activity of dopaminergic cell bodies in the POA or by reducing DA release from PIT nerve terminals which originate from these cells. Since pheromone-induced changes in DA content were not observed in the HYP, the effects of 17,20B-P on DA turnover appears to be primarily associated with a reduction of DA release from nerve terminals rather than changes in DA synthesis. Rapid pheromonal effects on DA release may be biologically relevant if one considers the latency of the male GtH response to water-borne 17,20B-P. In this and earlier studies (see: Chapter 5), males consistently exhibit a 2-3 fold increase in blood GtH within 15 min of exposure to the pheromone. The latency of the pheromone-induced response could be explained by a rapid abatement of DA release to the PIT without invoking possible reductions in DA synthesis. However, possible long-term effects of 17,20B-P exposure on DA synthesis cannot be ruled out, since chronic exposure to the pheromone increases GtH for at least 8-12 h (Chapter 5). This final point stresses the need to examine more accurately the effects of water-borne 17,20B-P on DA synthesis and release in male goldfish.

Changes in brain and PIT concentrations of sGnRH and cGnRH-II were not observed in pheromone-exposed males, suggesting that these forms of GnRH are not involved in mediating the GtH response to water-borne 17,20B-P. However, simultaneous decreases in sGnRH concentrations have been observed in the OB, TEL, HYP, and PIT of spontaneously ovulating goldfish, suggesting that the brain sGnRH system functions as an integrated unit for the activation of GtH secretion during ovulation (Yu et al., 1987). Similar reductions in brain and PIT levels of GnRH have been reported during natural spawning in both male and female roach, *Rutilus rutilus* (Breton et al., 1988a, 1988b). In contrast, the performance of spawning behavior significantly increases levels of serum GtH and sGnRH concentrations in the OB, TEL and HYP, but not the PIT in male goldfish (K.L. Yu, personal communication). Although these findings suggest that sexual interaction can influence brain GnRH levels and GtH release in goldfish and other teleosts, it is unclear why male and female goldfish show opposite GnRH changes in the same brain areas and whether these changes are directly responsible for causing increased GtH release during ovulation or following the performance of male sexual behavior. However, unlike the rapid GtH and DA responses to water-borne 17,20B-P which occurred within 20 min of exposure, the GnRH changes described for ovulatory goldfish occurred over a period of 2-4 days (Yu et al., 1987) while those of roach occurred over several weeks (Breton et al., 1988a; 1988b). Although the possible involvement of GnRH cannot be ruled out (see below), the present results indicate that the neuroendocrine trigger for 17,20B-P-induced GtH release involves a rapid reduction of DA turnover rather than a direct stimulation of GnRH release to the PIT.

Although a reduction in DA inhibition alone (Omeljaniuk et al., 1987) may account for male GtH responses to water-borne 17,20B-P, reduced dopaminergic input to the PIT may allow endogenous GnRH to stimulate GtH release (see review: Peter et al., 1986). In other words, an abatement of DA turnover in the PIT may result in a disinhibition of GnRH neuronal activity allowing for enhanced GnRH production and/or potentiated GnRH release to the PIT. The experiments which examined the effects of DA synthesis blockers on the

GtH response to 17,20B-P support this possibility. For example, injections of MPT and CARB significantly reduced DA content in the PIT (Experiments 4 and 5), whereas MPT, but not CARB, reduced DA levels in the HYP (Experiment 4). Since the PIT lies outside the blood-brain barrier in goldfish (Peter et al., 1989), the actions of these drugs are consistent with their abilities to cross the blood-brain barrier (Gilman et al., 1985). In addition, CARB specifically decreased DA levels in the PIT, presumably by interrupting DA synthesis, since concentrations of NA and 5HT were not affected by the drug (Experiment 5). Moreover, CARB-injected 17,20B-P-exposed males had significantly higher levels of blood GtH than did saline-injected 17,20B-P-exposed males (Experiments 4 and 5). Although the combination of CARB and 17,20B-P may have had additive effects on reducing DA turnover in the PIT and increasing GtH release, the drug-treated pheromone-exposed males had significantly higher levels of GnRH in the PIT than did saline-injected pheromone-exposed males (Experiment 5). These results indicate that alterations in DA turnover can affect GnRH levels in the PIT and suggest that the GtH response to water-borne 17,20B-P may be regulated through the combined actions of DA and GnRH.

Studies in mammals indicate that a number of amine neurotransmitters (NA, DA and 5HT) are involved in the regulation of GtH release, presumably by influencing the activity of GnRH neurons which project to the median eminence (see reviews: Klara and Klara, 1983; Barraclough and Wise, 1982). Although the involvement of DA in the regulation of GnRH release remains controversial, there is evidence to suggest that DA functions to inhibit GnRH release to the median eminence (Barraclough and Wise, 1982). In addition, recent studies indicate that catecholamines modulate the release of GnRH in goldfish; NA and A stimulate GnRH neurons centrally, whereas DA inhibits GnRH neurons centrally as well as at the level of the terminals in the PIT (Peter et al., 1989). Based on these findings and the results of the present study, it is proposed that water-borne 17,20B-P causes an immediate reduction in DA turnover in the PIT which influences GtH both directly and indirectly through modulation of GnRH input to the PIT.

The possible involvement of DA and GnRH in regulating GtH responses to water-

borne 17,20B-P are incorporated in a tentative model of the 17,20B-P pheromone system in male goldfish (Fig. 8.6). The rapid reduction of DA turnover in the PIT appears to serve as the neuroendocrine trigger for 17,20B-P-induced GtH release in male goldfish. Although speculative, pheromone-induced reductions in DA inhibition may allow GnRH to maintain increased levels of GtH for extended periods of time, thus ensuring sufficient increases in milt production by the time of spawning (Dulka et al., 1987a). The utility of this model suggests that it could play an important role in determining the relative involvement of DA and GnRH as mediators of the male GtH response to water-borne 17,20B-P in goldfish. In addition, the 17,20B-P pheromone system in goldfish serves as an excellent comparative model to study the neuroendocrine regulation of vertebrate endocrine responses to sex pheromones in general.

Figure 8.0. Planes of cuts used to dissect the goldfish brain into different regions for determination of catecholamine and GnRH content. OB, olfactory bulbs; TEL, telencephalon; HYP, hypothalamus; PIT, pituitary.

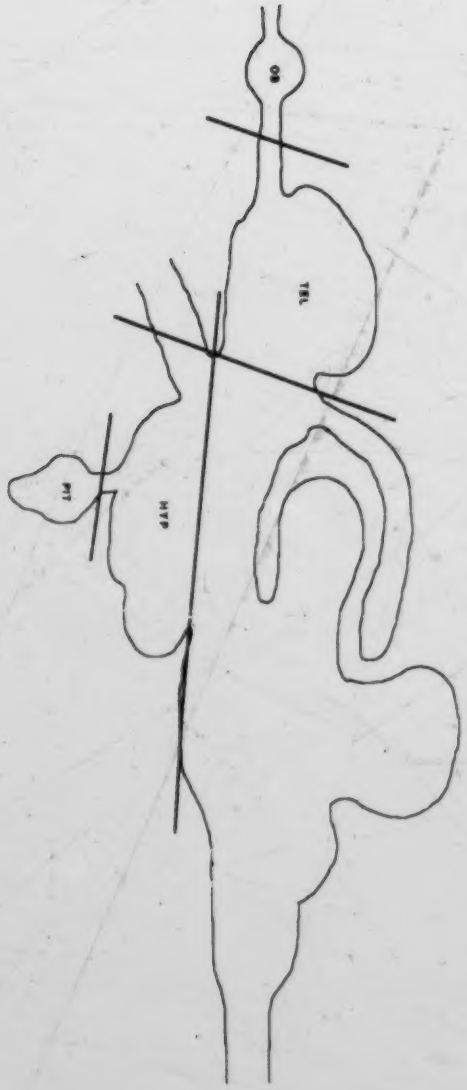


Figure 8.1A. Serum GtH levels (X ± SEM) in male goldfish following exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars) for the times indicated. Significant differences between ethanol and 17,20B-P-exposed groups at each sample time are indicated by asterisks ($p<0.05$, Least Squares Means analysis). n=6 in all groups.

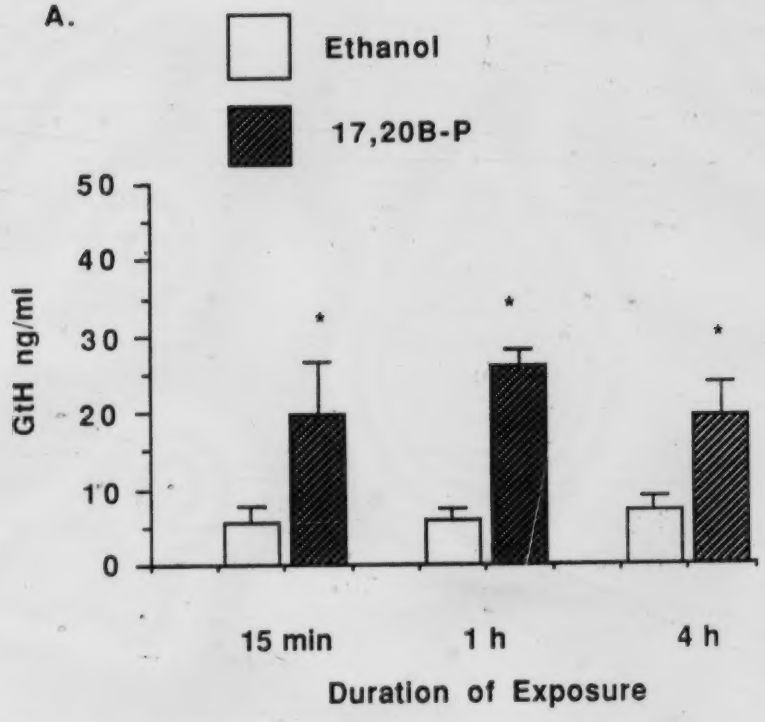

A.
Ethanol
17,20B-P
GtH ng/ml
50
40
30
20
10
0
*
*
*
15 min
1 h
4 h
Duration of Exposure

Figure 8.1B. Levels of catecholamines (X ± SEM) measured in the olfactory bulbs of male goldfish following exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars) for the times indicated. Animals are those in figure 8.1A. n=6 in all groups. Abbreviations: NA, noradrenaline; DA, dopamine; 5HT, serotonin; DOPAC, dihydroxyphenylacetic acid; 5HIAA, 5-hydroxyindoleacetic acid. ND: not determined.

B. OLFACTORY BULBS

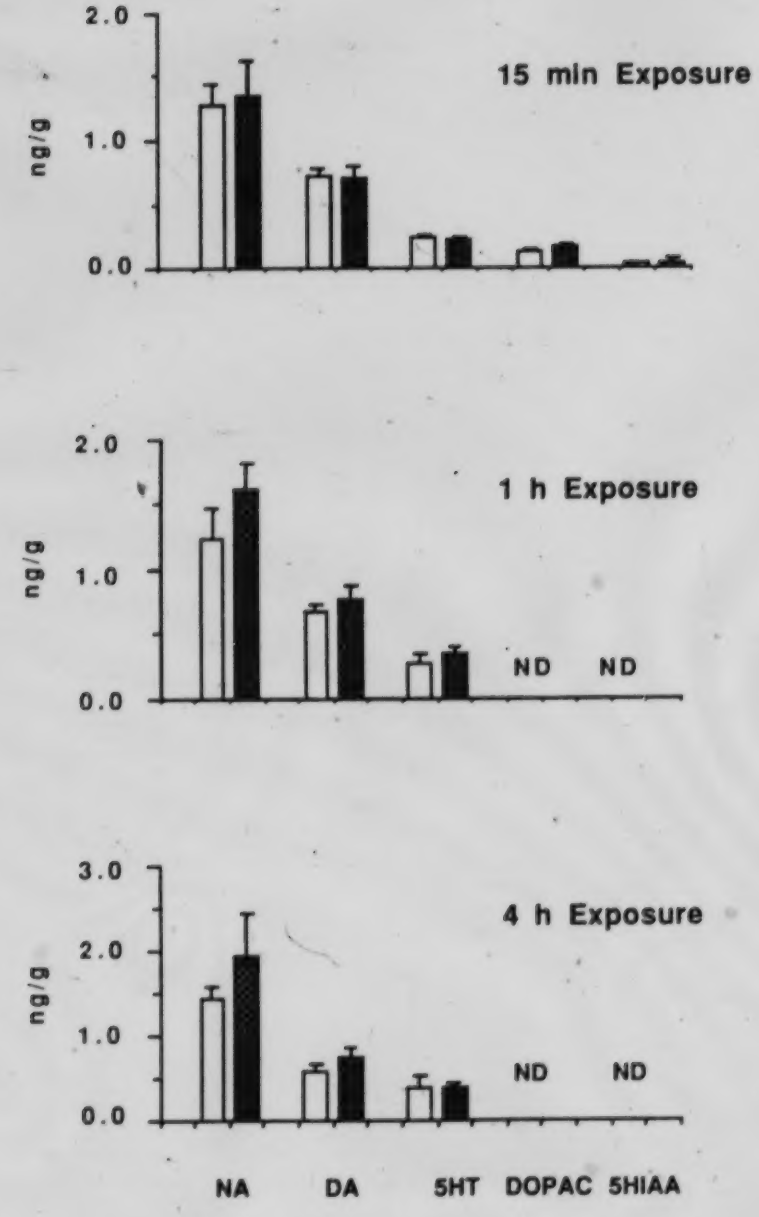


Figure 8.1C. Levels of catecholamines (X ± SEM) measured in the telencephalon of male goldfish following exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars) for the times indicated. Animals are those in figure 8.1A. n=6 in all groups. Abbreviations: NA, noradrenaline; DA, dopamine; 5HT, serotonin; DOPAC, dihydroxyphenylacetic acid; 5HIAA, 5-hydroxyindoleacetic acid. ND: not detected.

C. TELENCEPHALON

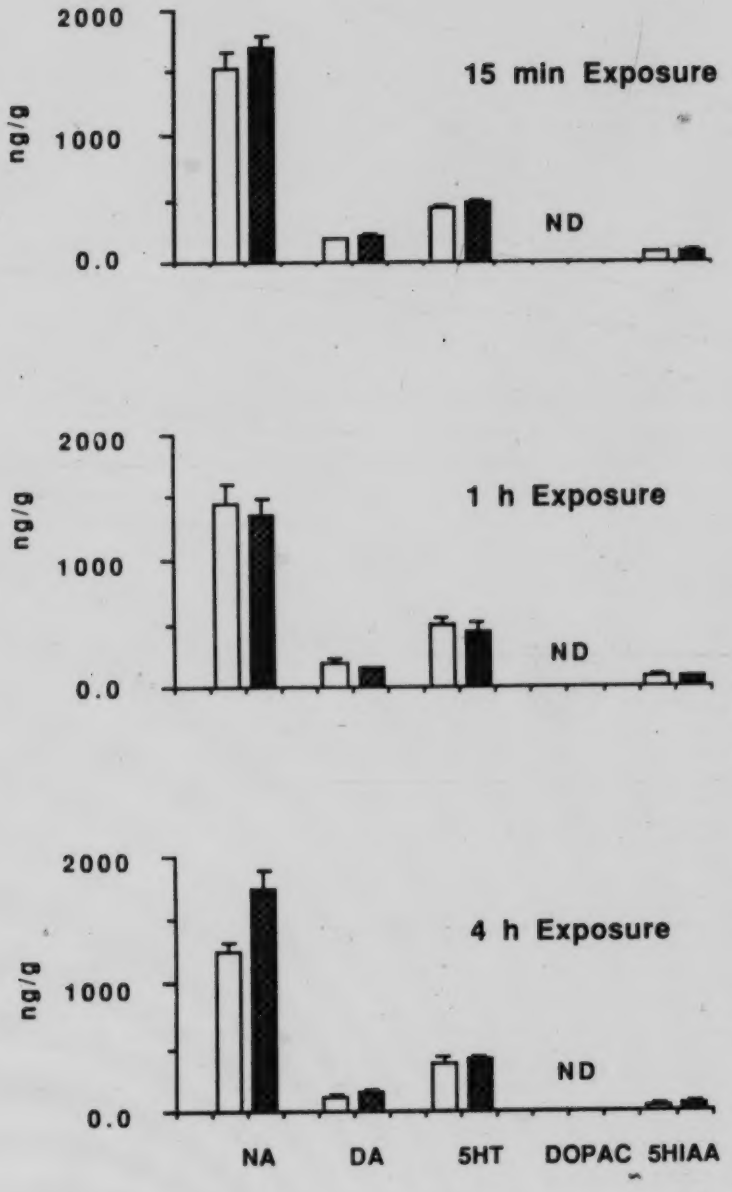


Figure 8.1D. Levels of catecholamines (X ± SEM) measured in the hypothalamus of male goldfish following exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars) for the times indicated. Animals are those in figure 8.1A. n=6 in all groups. Abbreviations: NA, noradrenaline; DA, dopamine; 5HT, serotonin; DOPAC, dihydroxyphenylacetic acid; 5HIAA, 5-hydroxyindoleacetic acid.

D. HYPOTHALAMUS

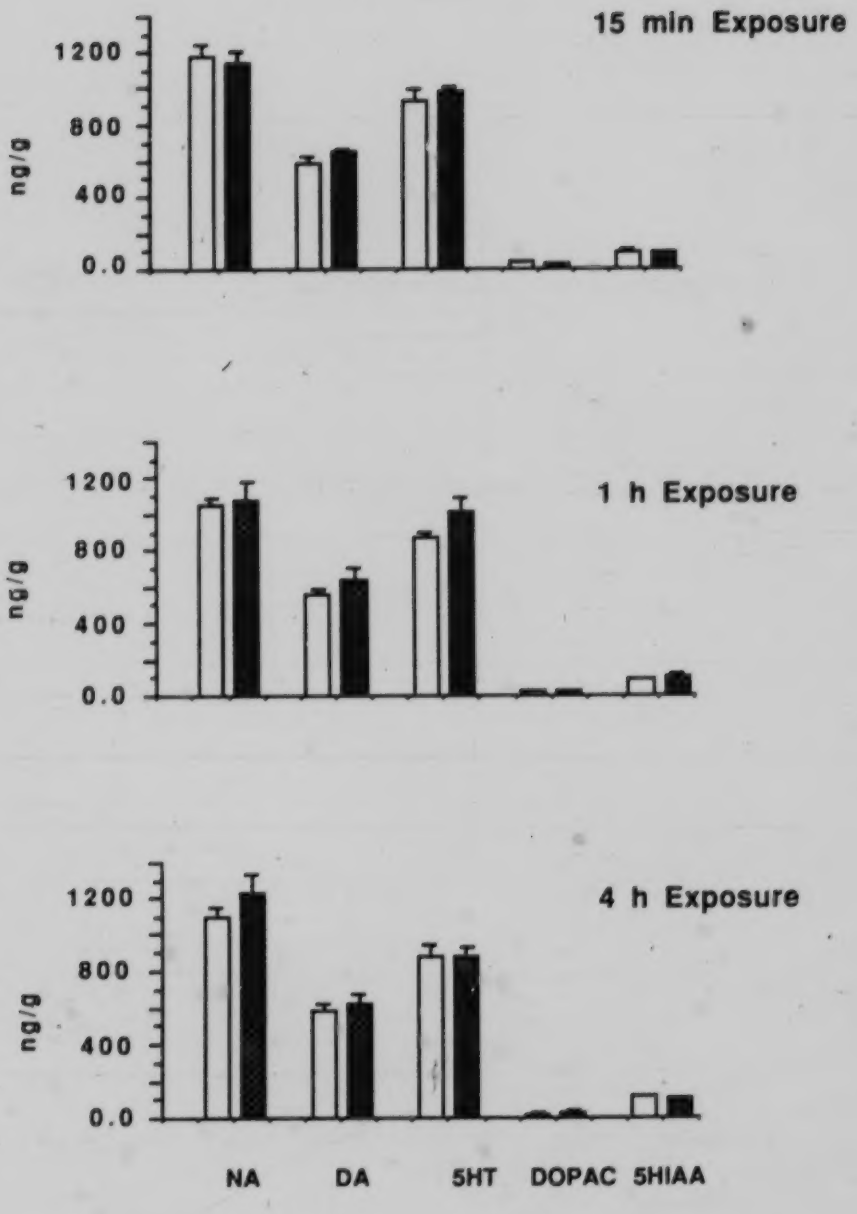


Figure 8.1E. Levels of DA and DOPAC (X ± SEM) measured in the pituitary of male goldfish following exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars) for the times indicated. Animals are those in figure 8.1A. n=6 in all groups.

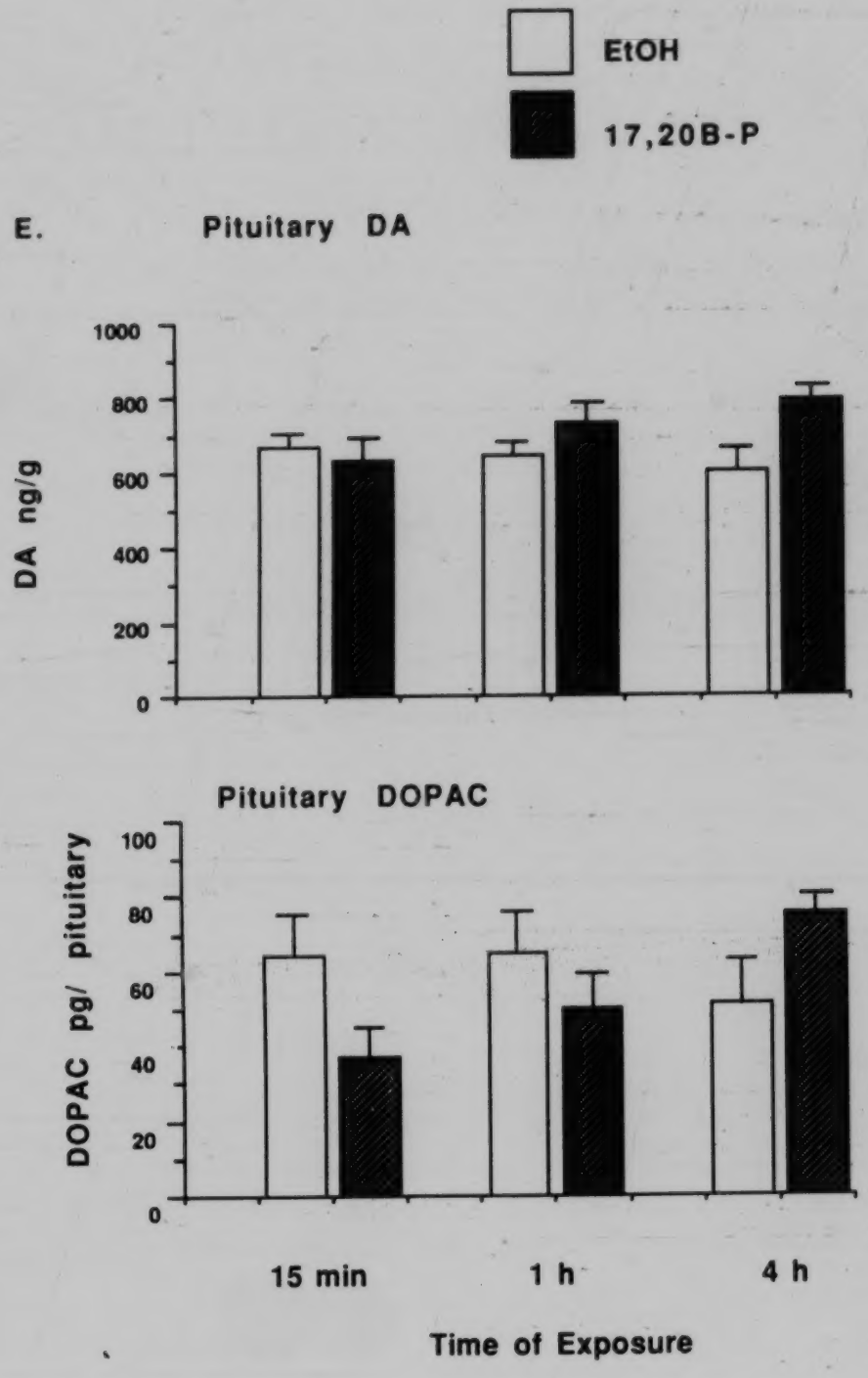

EtOH
17,20B-P
E.
Pituitary DA
DA ng/g
1000
800
600
400
200
0
Pituitary DOPAC
DOPAC pg/ pituitary
100
80
60
40
20
0
15 min
1 h
4 h
Time of Exposure

Figure 8.2A-D. Levels (X ± SEM) of serum GtH (**A**) and pituitary DA (**B**), DOPAC (**C**) and the ratio of DOPAC to DA (**D**) in male goldfish following exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars) for the times indicated. Significant differences between the ethanol and 17,20B-P-exposed groups are indicated by asterisks (* $p<0.05$, ** $p<0.01$; 2-way ANOVA, Least Squares Means analysis). n=18 in all groups.

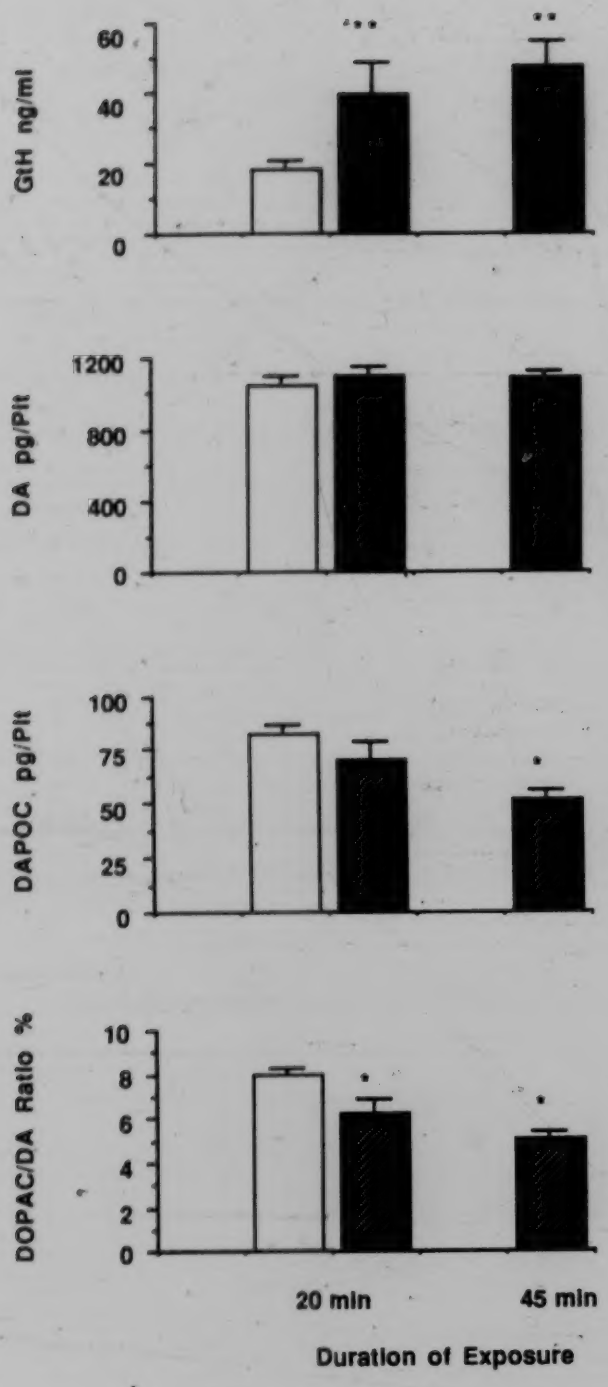

A. GtH
GtH ng/ml
60
40
20
0
***
**
B. DA
DA pg/Plt
1200
800
400
0
C. DOPAC
DAPOC pg/Plt
100
75
50
25
0
*
D. DOPAC/DA
DOPAC/DA Ratio %
10
8
6
4
2
0
*
*
20 min
45 min
Duration of Exposure

Figure 8.3A-D. Levels (X ± SEM) of serum GtH (A) and pituitary DA (B), DOPAC (C) and the ratio of DOPAC to DA (D) in male goldfish following exposure to either nothing (lightly shaded bars), 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (darkly shaded bars) for the times indicated. Significant differences between the ethanol and 17,20B-P-exposed groups are indicated by asterisks (* $p<0.05$, ** $p<0.01$; 2-way ANOVA, Least Squares Means analysis). n=15 in groups exposed for 0 and 45 min whereas n=9 in groups exposed for 2h.

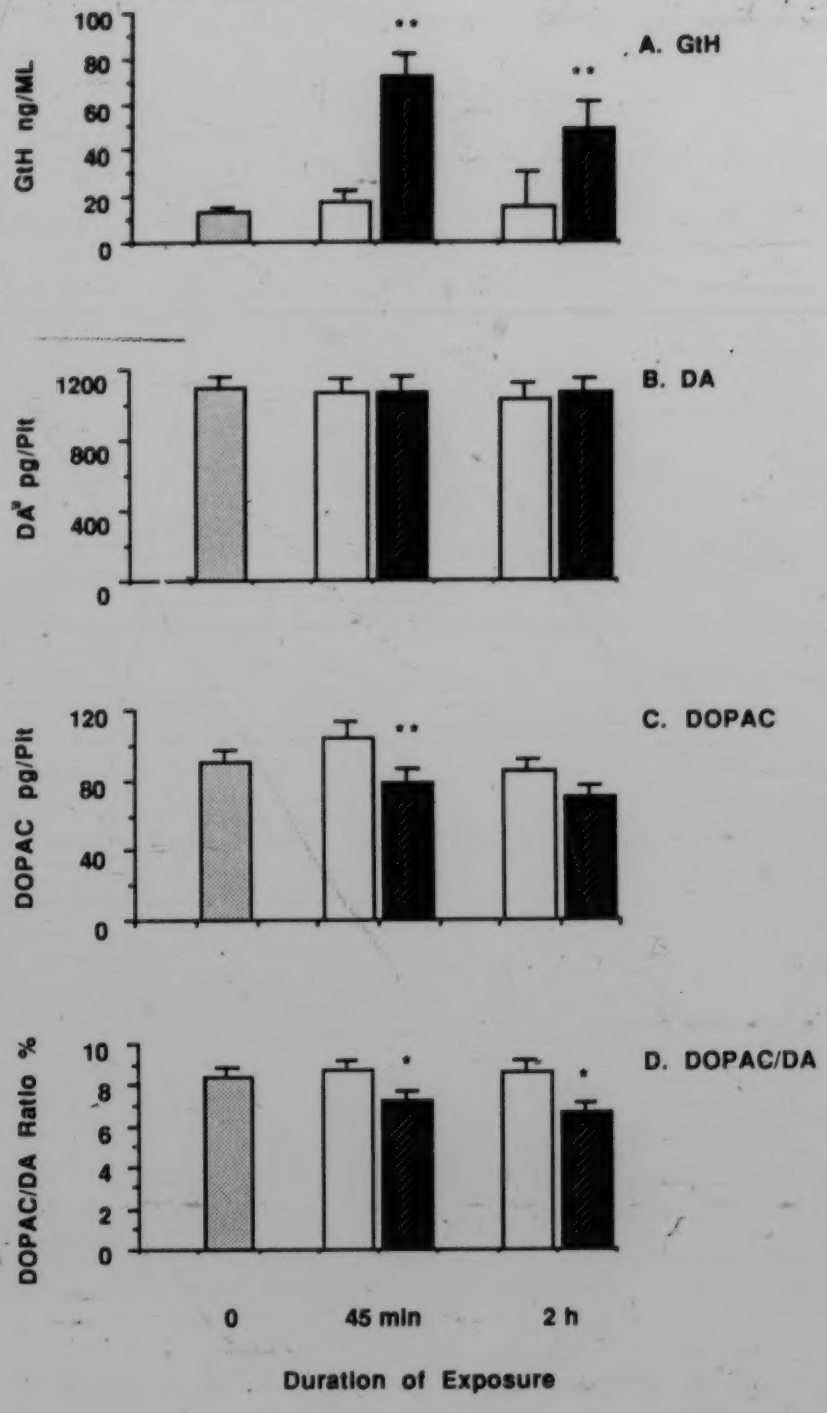
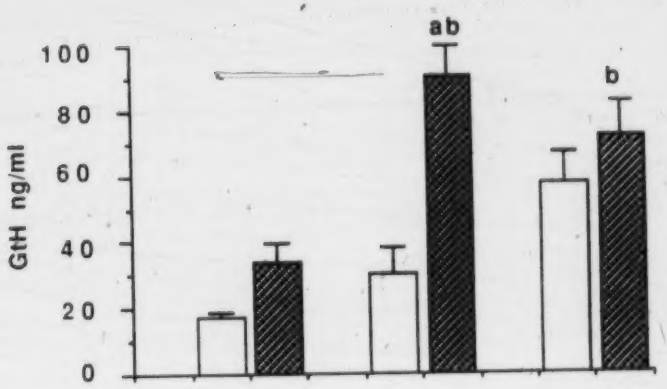
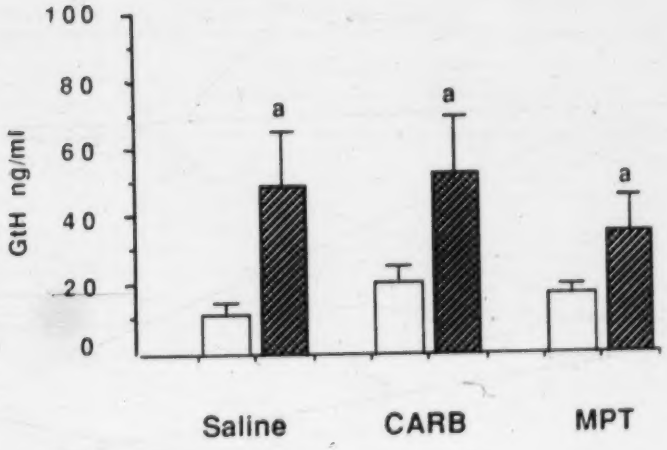

A. GtH
GtH ng/ML
100
80
60
40
20
0
**
**
B. DA
DA pg/Plt
1200
800
400
0
C. DOPAC
DOPAC pg/Plt
120
80
40
0
**
D. DOPAC/DA
DOPAC/DA Ratio %
10
8
6
4
2
0
*
*
0
45 min
2 h
Duration of Exposure

Figure 8.4A-B. Effects of carbidopa (CARB, 50 µg/g Bd. Wt.) and α-methyl-para-tyrosine (MPT, 300 µg/g Bd. Wt.) on male GtH levels (X ± SEM) following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). Exposures in **A** (13 h drug; 12 h exposure) began 1 h after the fish were injected with either saline or carbidopa. All exposures in **B** (17 h drug; 4 h exposure) lasted for 4 h beginning 12 h after the fish were injected with the drugs. Letters above the bars denote differences among groups as indicated below the figure. n=4 in all groups.

A. 13 h Drug; 12 h Exposure



B. 17 h Drug; 4 h Exposure

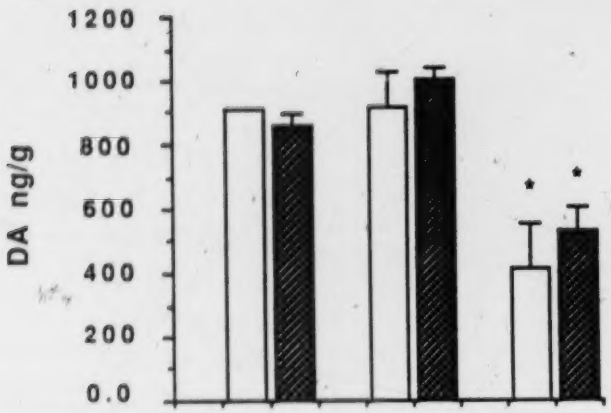


a. $p<0.05$ compared to saline+ethanol group

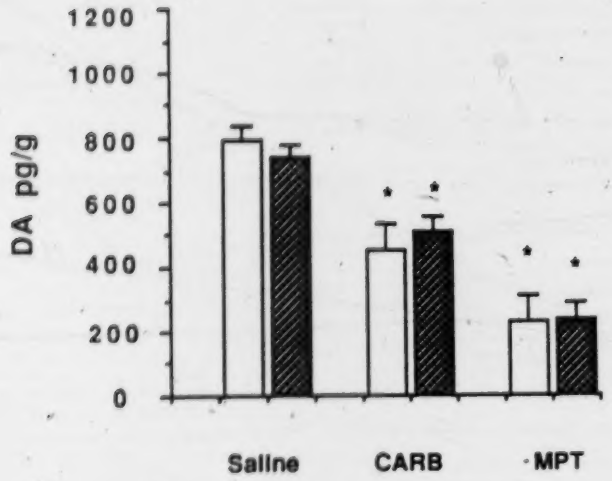
b. $p<0.05$ compared to saline+17,20B-P group

Figure 8.4C-D. Effects of carbidopa (CARB, 50 µg/g Bd. Wt.) and α-methyl-para-tyrosine (MPT, 300 µg/g Bd. Wt.) on hypothalamic and pituitary levels (X ± SEM) of DA in male goldfish following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). All exposures began 1 h after the fish were injected with either saline or carbidopa. Fish are those in A of figure 8.2A-B. All other conventions as in figure 8.2A-B.

C. HYPOTHALAMUS



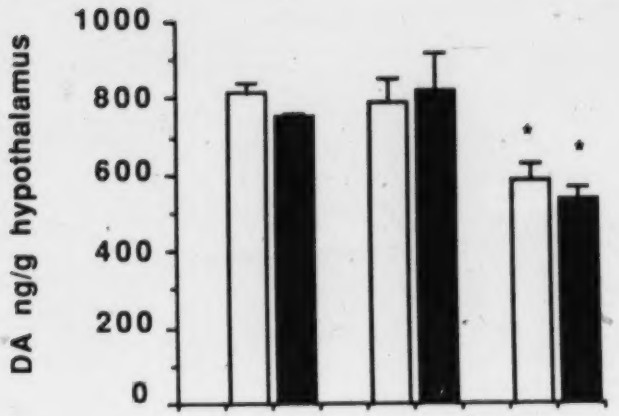
D. PITUITARY



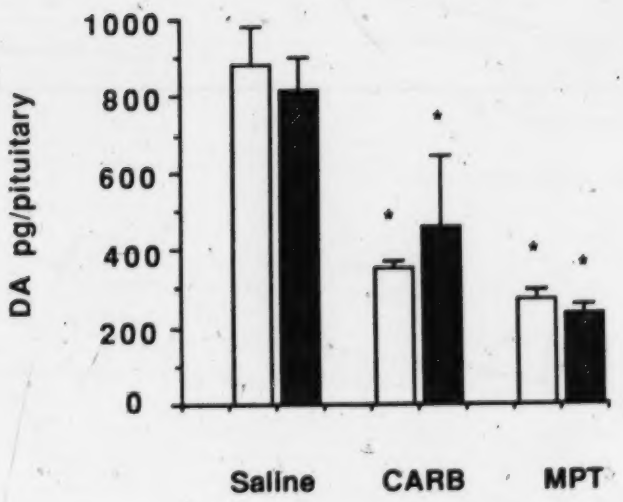
(13 h drug; 12 h 17,20-P)

Figure 8.4E-F. Effects of carbidopa (CARB, 50 µg/g Bd. Wt.) and α-methyl-para-tyrosine (MPT, 300 µg/g Bd. Wt.) on hypothalamic and pituitary levels (X ± SEM) of DA in male goldfish exposed to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). All exposures lasted for 4h beginning 12 h after the fish were injected with the drugs Fish are those in **B** of figure 8.2A-B. All other conventions as in figure 8.2A-B.

E. HYPOTHALAMUS

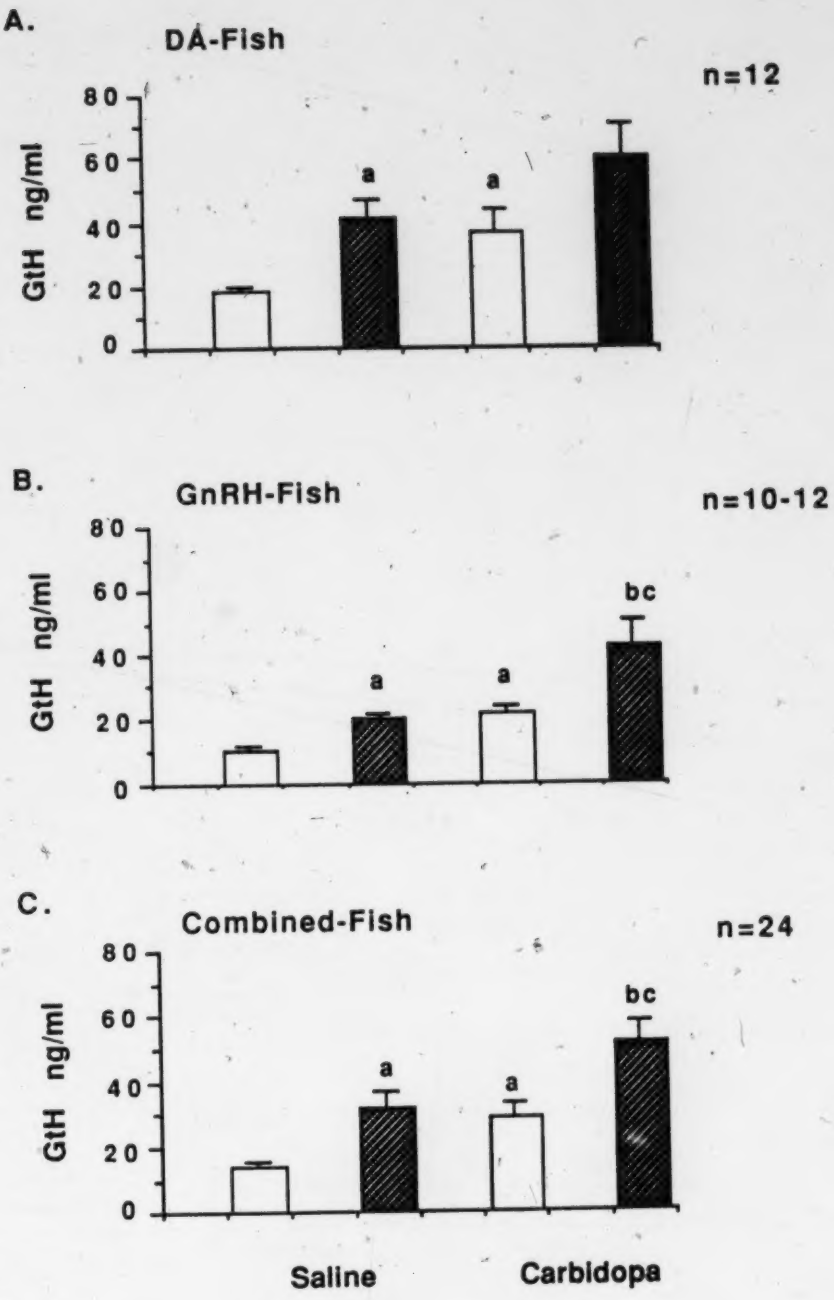


F. PITUITARY



(17 h drug; 4 h 17,20B-P)

Figure 8.5A-C. Effect of carbidopa (50 µg/g Bd. Wt.) on male GtH levels (X ± SEM) following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). All exposures began 1 h after the fish were injected with either saline or carbidopa. Brain and pituitary samples from fish in **A** (DA-Fish) were processed for measurement of catecholamines by HPLC. Brain and pituitary samples from fish in **B** (GnRH-Fish) were processed for measurement of sGnRH and cGnRH-II levels by RIA. The GtH levels in **C** (Combined-Fish) represent the combined averages of the corresponding groups in **A** and **B**. Letters above the bars denote differences among groups as indicated below the figure. Sample sizes are indicated to the right of the figures.



a. different from saline+ethanol
b. different from CARB+ethanol
c. different from saline+17,20B-P

Figure 8.5D-F. Effects of carbidopa (50 µg/g Bd. Wt.) on NA (D), 5HT (E) and DA (F) levels (X ± SEM) in the hypothalamus of male goldfish following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). Control fish were injected with saline (5.0 µl/g Bd. Wt.). All exposures began 1 h after the fish were injected. Animals are those in A of figure 8.3A-C. Abbreviations: NA, noradrenaline; DA, dopamine; 5HT, serotonin.

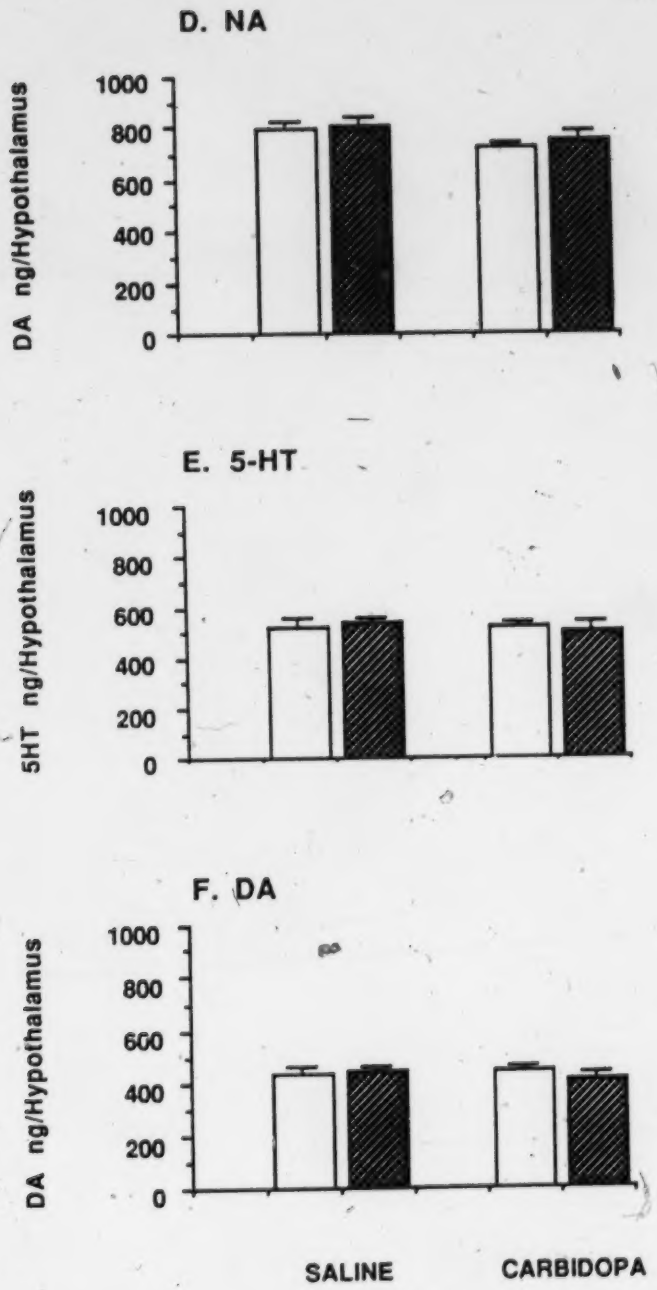
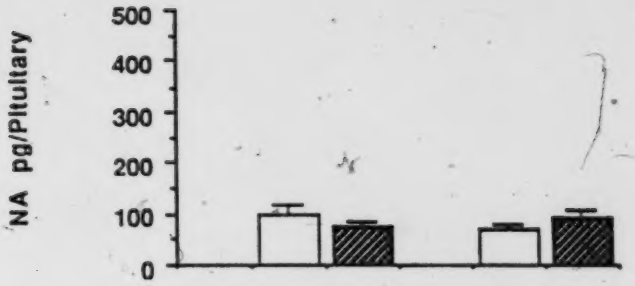

D. NA
DA ng/Hypothalamus
1000
800
600
400
200
0
E. 5-HT
5HT ng/Hypothalamus
1000
800
600
400
200
0
F. DA
DA ng/Hypothalamus
1000
800
600
400
200
0
SALINE
CARBIDOPA

Figure 8.5G-I. Effects of carbidopa (50 µg/g Bd. Wt.) on NA (G), 5HT (H) and DA (I) levels (X ± SEM) in the pituitary of male goldfish following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). Control fish were injected with saline (5.0 µl/g Bd. Wt.). All exposures began 1 h after the fish were injected. Animals are those in A of figure 8.3A-C. Abbreviations: NA, noradrenaline; DA, dopamine; 5HT, serotonin.

G. NA


NA pg/Pitultary
500
400
300
200
100
0

H. 5-HT

5-HT pg/Pitultary
500
400
300
200
100
0

I. DA

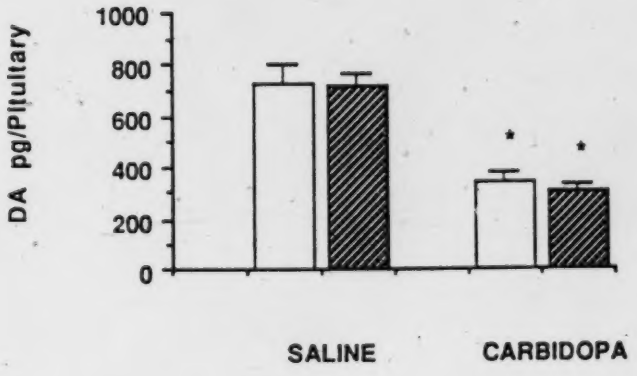

DA pg/Pitultary
1000
800
600
400
200
0
*
*
SALINE
CARBIDOPA

Figure 8.5J-L. Effects of carbidopa (50 µg/g Bd. Wt.) on sGnRH levels (X ± SEM) in the telencephalon (**J**), hypothalamus (**K**) and pituitary (**L**) of male goldfish following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). All exposures began 1 h after the fish were injected with either saline or carbidopa. Animals are those in **B** of Figure 8.3A-C.

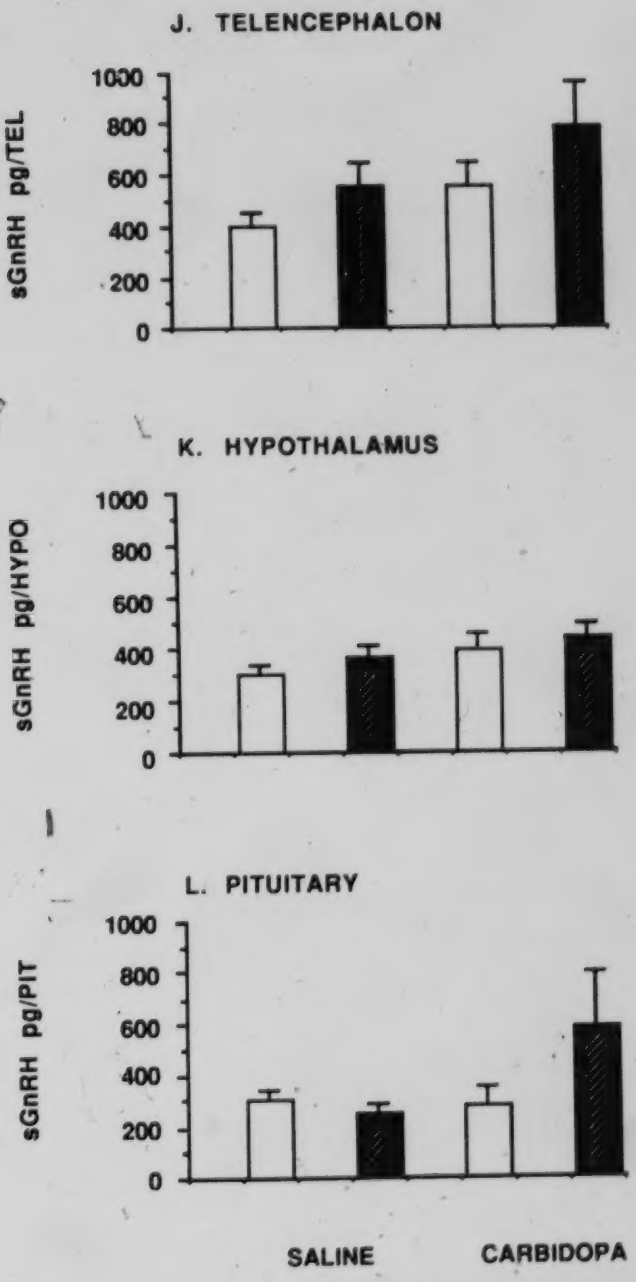
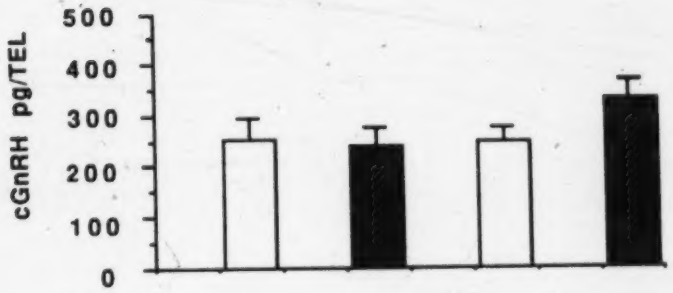
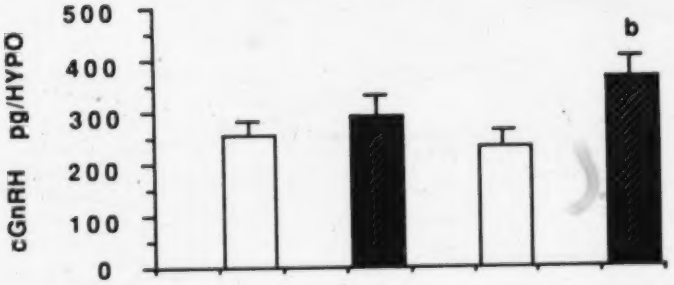

J. TELENCEPHALON
sGnRH pg/TEL
1000
800
600
400
200
0
K. HYPOTHALAMUS
sGnRH pg/HYPO
1000
800
600
400
200
0
L. PITUITARY
sGnRH pg/PIT
1000
800
600
400
200
0
SALINE
CARBIDOPA

Figure 8.5M-O. Effects of carbidopa (50 µg/g Bd. Wt.) on cGnRH-II levels (X ± SEM) in the telencephalon (**M**), hypothalamus (**N**) and pituitary (**O**) of male goldfish following 12 h of exposure to either 100 µl ethanol (Clear bars) or $5X10^{-10}$ M 17,20B-P (Shaded bars). All exposures began 1 h after the fish were injected with either saline or carbidopa. Fish are those in **B** of Figure 8.3A-C. Letters above the bars indicate significant differences among groups: b=$p<0.05$ compared to the carbidopa+ethanol group; c=$p<0.05$ compared to the saline+17,20B-P group (2-way ANOVA, Least Squares Means analysis).

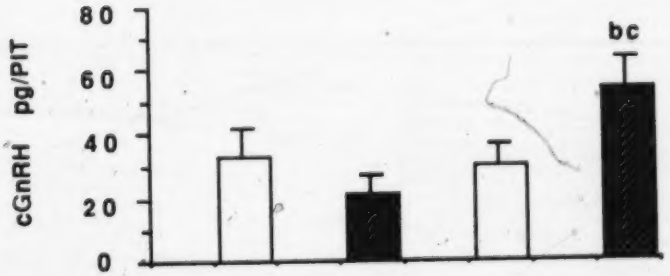
M. TELENCEPHALON



N. HYPOTHALAMUS

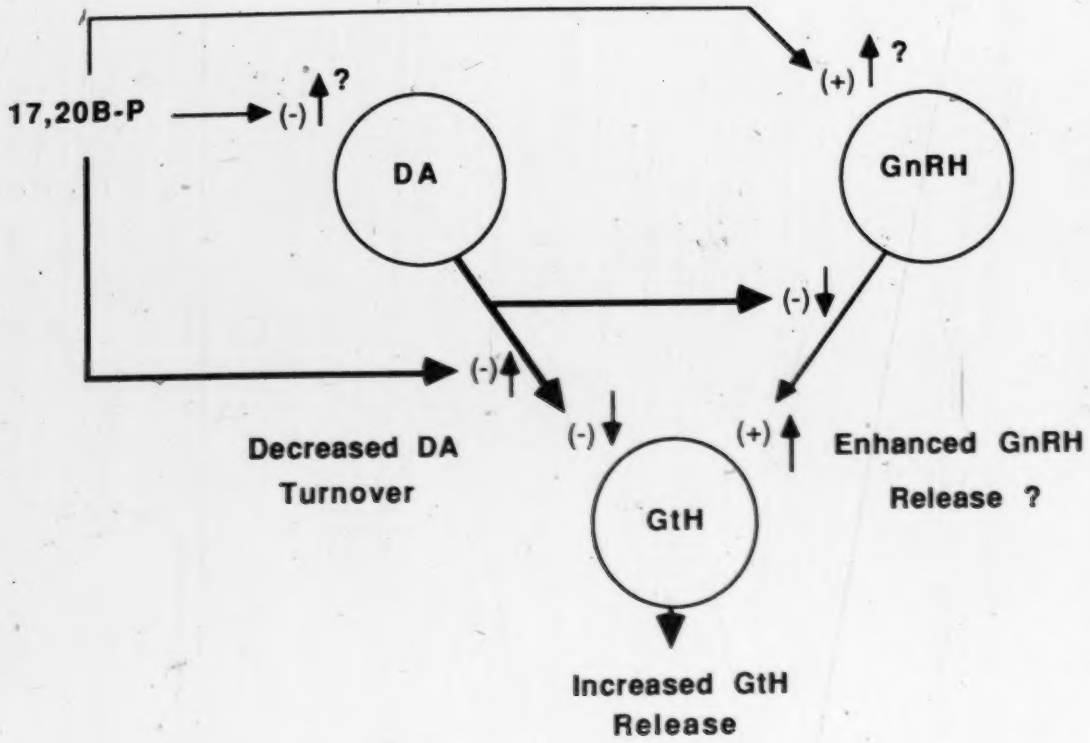


O. PITUITARY



SALINE CARBIDOPA

Figure 8.6. Speculative model on the neuroendocrine regulation of the gonadotropin (GtH) response to water-borne 17,20B-P in male goldfish. A rapid reduction of dopamine (DA) turnover appears to serve as the neuroendocrine trigger for 17,20B-P-induced GtH release (thick lines). However, reductions in DA turnover may enhance gonadotropin-releasing hormone (GnRH) release to the pituitary (thin lines). (-) and (+) indicate inhibition and stimulation, respectively. Small arrows adjacent to these symbols represent possible shifts in the mode of action of either DA or GnRH on GtH release. Question marks indicate hypothetical projections and possible physiological affects.


17,20B-P
(-) ?
DA
(+) ?
GnRH
(-)
(-)
Decreased DA
Turnover
(-)
(+)
Enhanced GnRH
Release ?
GtH
Increased GtH
Release

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9. GENERAL DISCUSSION

Although behavioral responses to putative sex pheromones have been extensively investigated in teleosts (for review: Chapter 1), there have been few investigations on physiological responses to sex pheromones. A major problem in understanding pheromone function in teleosts has been a lack of information on pheromone identities. However, recent studies of goldfish provide strong evidence that 17,20B-P, the steroidal hormone which promotes oocyte final maturation (Nagahama et al., 1983), is released to the water by preovulatory females where it functions as a potent sex pheromone to stimulate male GtH release and increased milt production (Stacey and Sorensen, 1986; Dulka et al., 1987a; Sorensen et al., 1987). The preceding chapters of this thesis present original information on the male endocrine response to water-borne 17,20B-P in goldfish. This chapter will summarize and discuss the major findings of the thesis, and provide the basis for a model to study vertebrate sex pheromone function in general.

Release of 17,20B-P to the water by preovulatory females was described in Chapter 2. These results have been confirmed by Stacey et al. (1989) who provided a more accurate description of the pattern of 17,20B-P release by periovulatory females (see: appendix II). Together, these findings clearly demonstrate that preovulatory females begin to release 17,20B-P to the water approximately 10-12 h prior to ovulation. In addition, males exposed to water-borne 17,20B-P exhibit increases in blood GtH within 15 min and milt production within 4-6 h (Chapters 2 and 5). The 17,20B-P-induced increase in milt production is likely mediated by the acute rise in GtH since hypophysectomy blocks the milt response to the pheromone (Chapter 2). Both the temporal pattern of 17,20B-P release from preovulatory females and the latency of the milt response to water-borne 17,20B-P in males support the proposal that 17,20B-P is a pheromone which synchronizes milt production with ovulation in goldfish (Stacey and Sorensen, 1986; Dulka et al., 1987a).

Implicit in this proposal is the assumption that a pheromone-induced increase in milt production leads to an increase in male fertility, presumably by affecting the quantity and/or

quality of releasable milt. For example, water-borne 17,20B-P may increase male fertility by simply increasing the number of sperm that can be released at spawning or by promoting increased sperm motility within the water, as recently demonstrated in salmonids by Yamauchi et al. (1989). Although not directly tested in the present study, a pheromone-induced increase in male fertility could benefit both sexes in that males with elevated levels of milt would be expected to have a greater chance at fertilizing eggs, as would females if they spawn with 17,20B-P-exposed males. A high degree of reproductive synchrony between the sexes may be especially important for females, since ovulated eggs remain viable for only a few hours (N.R. Liley, personal communication) and therefore must be released soon after ovulation. Thus, evolutionary pressures may have selected for a mechanism which allows females to signal that ovulation is imminent. Conversely, selection pressures on males may have played a greater role in the evolution of the 17,20B-P pheromone system in goldfish. For example, because females normally spawn with groups of males, it is possible that the presence of fertile males has never been a factor limiting female reproductive success. Rather, the pheromonal function of 17,20B-P may have evolved as a mechanism allowing males increased success at sperm competition. Since 17,20B-P functions endogenously to promote oocyte final maturation (Nagahama et al., 1983; Goetz, 1983), its use as a female hormone was probably preexisting and later became adapted for pheromonal function only after males developed means of associating its release with impending ovulation. Presumably, selection pressures favored those males which developed olfactory receptors and neuroendocrine mechanisms which mediate the milt response to water-borne 17,20B-P.

The olfactory pathways by which water-borne 17,20B-P stimulates GtH release and milt production were examined in Chapter 3. The results support the earlier finding (Stacey and Sorensen, 1986) that lesions of the MOTs, but not the LOTs, abolish the milt response to water-borne 17,20B-P, and provide additional evidence that the pheromone-induced increase in milt production is mediated by elevated GtH release in male goldfish. In addition, the confirmation that both the GtH and milt responses are dependent on the MOTs support the hypothesis that this olfactory subdivision is involved in mediating responses to

pheromones in goldfish and other teleosts (Doving and Selset, 1980; Kyle et al., 1987; Resink, 1989).

As discussed in Chapter 3, the central projections of the MOTs terminate in mid-line regions of the ventral forebrain (Von Bartheld et al., 1984; Levine and Dethier, 1985; Springer, 1983) which have been implicated in the control of a variety of reproductive functions in goldfish and other teleosts (Demski and Hornby, 1982; Kyle et al., 1987). These regions include those that control male reproductive behavior (Kyle et al., 1982; Kyle and Peter, 1982; Koyama et al., 1984), sperm release (Demski and Hornby, 1982) and, more importantly, regions in the preoptic area (POA) that regulate GtH release (Peter et al., 1986). GtH secretion in goldfish is believed to be controlled through the combined actions of gonadotropin-releasing-hormone (GnRH) and dopamine (DA) which stimulate and inhibit GtH release, respectively (Peter et al., 1986). Because the POA contains high concentrations of both GnRH and DA cell bodies which project to the pituitary (Kah et al., 1986; Kah et al., 1987), a direct MOT projection to the POA may constitute a functional neuroanatomical pathway by which 17,20B-P exerts its action to stimulate GtH release and milt production in goldfish. Circumstantial evidence that olfactory information can reach the POA directly is provided by investigations which demonstrate that electrical stimulation of the OTs cause both sperm release (Demski and Northcutt 1983; Demski and Dulka, 1984) and depletion of neurosecretory material from cell bodies within the POA (Jasinski et al., 1967; Peter and Gorbman, 1968). The recent finding that exposure to 17,20B-P causes a reduction in the rate of DA turnover in the pituitary (Chapter 8), presumably by altering the activity of DA neurons in the POA, supports the proposal that a functional olfactory-preoptic pathway is involved in mediating GtH responses to water-borne 17,20B-P.

The olfactory sensitivity of males to various concentrations of water-borne 17,20B-P was examined in Chapter 4. The threshold water concentration necessary to induce a GtH response (approximately 10^{-11} M) is similar to that which evokes increased electrical activity in olfactory receptors as determined by EOG (10^{-12}-10^{-11} M; Sorensen et al., 1986; Sorensen et al., 1989). However, unlike dose-dependent EOG responses (Sorensen

et al., 1986; Sorensen et al., 1989), males do not show a dose-dependent increase in GtH following exposure to increasing, supra-threshold concentrations of water-borne 17,20B-P: (i.e. higher concentrations of 17,20B-P were no more effective at increasing GtH than were threshold concentrations). These results suggest that if males have graded responses to the pheromone they occur over a relatively small concentration range (i.e. 10^{-12}-10^{-11} M). However, as discussed in Chapter 4, the actual concentration of water-borne 17,20B-P may not be critical once threshold concentrations are detected by the male. For example, the neural mechanisms regulating the male's endocrine response to 17,20B-P may have evolved simply to detect impending ovulation via a chemical signal which undoubtedly varies in amplitude depending on such factors as stage of the ovulatory surge (Stacey et al., 1989) and distance from the source (Sorensen and Stacey, 1989).

A number of questions regarding the release of 17,20B-P from females and the responses of males to the pheromone remain to be examined. For example, there is currently no information on whether females release 17,20B-P in a continuous or pulsatile manner, or whether males are capable of continuously detecting the water-borne pheromone. In this regard, the methods employed in this thesis to study male responses to water-borne 17,20B-P may not necessarily reflect the conditions which normally occur just before spawning. As discussed by Stacey et al (1989), males may encounter gradients of 17,20B-P instead of the "uniform" concentrations created in the present study. For example, recent calculations indicate that if females release 17,20B-P at a constant rate, the amount present in the water would be sufficient to produce only a small and short-lived odor plume which would be detectable only for a few seconds and, especially at moderate swimming speeds, only within a few centimeters of the female (Sorensen and Stacey, 1989). However, by actively chasing females, males may encounter many pheromone pulses. In this regard, there is a need to re-examine the behavioral response of males to water-borne 17,20B-P. As originally described by Sorensen et al. (1989a), males show increased levels of sexual arousal following exposure to the pheromone. Behavioral responses to 17,20B-P may allow males to keep in close physical contact with 17,20B-P-releasing females, until the time of ovulation. However, although males appear capable of

responding to brief pulses of 17,20B-P (see: Chapter 4), it is not known if repetitive pulses have additive effects on GtH and milt production.

The time-course of male endocrine responses to water-borne 17,20B-P was studied in Chapter 5. Exposure of males to water-borne 17,20B-P was found to stimulate GtH release within 15 min and to maintain elevated levels of GtH for at least 8-12 h. Pheromone-induced increases in GtH stimulate the production of 17,20B-P (presumably of testicular origin) within 15 min and cause increased milt production within 4-6 h of exposure. In contrast, elevations in serum GH occurred inconsistently and only after the elevations in milt volume had occurred. The results provide a detailed account of the functional endocrine events that underlie the milt response to water-borne 17,20B-P. I propose that the timing of these responses allows milt production to be synchronized with ovulation and spawning in goldfish.

As discussed in Chapter 5, male goldfish appear to respond to water-borne 17,20B-P in a reflexive manner. Detection of supra-threshold concentrations of the pheromone triggers a series of neuroendocrine and endocrine events which ultimately lead to increased milt production. Water-borne 17,20B-P appears to cause increased GtH release by reducing DA inhibition at the level of the pituitary (Chapter 8). Once this central reflex is activated, the resulting increase in GtH acts peripherally to stimulate the production of gonadal steroids which give rise to the milt response to water-borne 17,20B-P (Chapter 5). This pheromone system appears to be a novel example in which an external stimulus (i.e. water-borne 17,20B-P) gives rise to a neuroendocrine reflex in a teleost.

The reflex response to 17,20B-P in male goldfish appears analogous to situations in which chemosensory and/or somatosensory stimuli can cause reflex ovulation in some species of mammals. The involvement of chemosensory cues in mammalian reproduction has been well documented (see reviews: Muller-Schwartze and Silverstein, 1980); Allen and Adler, 1985). For example, in many rodents, the odor of adult males alone can delay puberty in juvenile males but accelerate puberty in juvenile females (*Vandenbergh Effect*, Vanderburgh, 1967, 1969), induce estrus and ovulation in previously anestrus adult

females (*Whitten Effect*, Whitten and Champlin, 1972), and/or cause failure of embryo implantation and the re-initiation of the estrus cycle in recently mated females (*Bruce Effect*, Bruce, 1960; Bruce and Parrot, 1960). All of the above effects are believed to be caused by male pheromones which operate through neuroendocrine mechanisms to affect the female reproductive cycle. Indeed male odors have been shown to elicit surges of GtH in female mice (Bronsen and Desjardin, 1974). In addition, the odor of male urine causes discrete changes in GnRH and NA concentrations in the olfactory bulbs of female mice and voles (Dluzen et al., 1981; Dluzen and Ramirez, 1983). In other cases, non-chemosensory cues can cause reflex ovulation. For example, in rabbits, peripheral stimulation (penile thrusting) of the vagina appears to be sufficient to cause reflex ovulation within one hour of copulation (see review: Allen and Adler, 1985). The sensory input of vaginal stimulation converges through the spinal cord to reach areas of the medial basal hypothalamus that control the ovulatory GtH surge. Vaginal stimulation induces increased multiunit activity in the arcuate, premammillary and posterolateral hypothalamic nuclei, whereas the same stimulation causes reduced activity in the medial POA and ventromedial hypothalamus (Allen and Adler, 1985). Electrical brain stimulation and lesion studies have verified the involvement of these areas in controlling the ovulatory GtH surge in rabbits and other mammalian species (see review: Allen and Adler, 1985). Thus, in both spontaneous and reflex ovulators, the peripheral nervous system conveys chemosensory and/or somatosensory stimuli to the central nervous system which transduces these stimuli into hormonal events by altering the functional activity of the hypothalamic-pituitary-gonadal axis. The mediobasal hypothalamus-median eminence complex is a final common pathway for both chemosensory and somatosensory initiated ovulatory reflexes in mammals. Although teleosts lack a median eminence-hypothalamic-pituitary portal system, they possess direct innervation of the anterior pituitary from hypothalamic regions that are considered to be homologous to those areas that project to the median eminence in mammals (Batten and Ingleton, 1987; Peter et al., 1989). Moreover, as for the GtH responses to water-borne 17,20B-P in goldfish, only brief periods (seconds to minutes) of chemosensory and/or somatosensory stimuli are required to induce both immediate and

long-term endocrine changes in reflex ovulators (Allen and Adler, 1985). Based on these similarities, the 17,20B-P pheromone system in goldfish serves as an excellent comparative model to study the sensory input, neuroendocrine regulation and biological significance of endocrine responses to sex pheromones in vertebrates (see below).

The experiments described in Chapter 6 examined whether males show a day-night difference in GtH responses to water-borne 17,20B-P. The experiments were conducted on the premise that males would be most responsive to 17,20B-P over periods when the pheromone is normally released by females (22:00-06:00 h; Stacey et al., 1989). However, the results clearly indicate that males are capable of responding to water-borne 17,20B-P at all times of the day. The lack of a circadian difference in endocrine sensitivity to 17,20B-P is clearly different from that exhibited by males exposed to spontaneously ovulated or PG-injected females (Chapter 7). Although males frequently exhibit increased levels of GtH following spawning (Kyle et al., 1985; Sorensen et al., 1989a), they show a marked nocturnal difference in endocrine responses to ovulated and PG-injected females, such that GtH responses are maximal during the scotophase and often absent during the photophase (Dulka et al., 1987b; N.E. Stacey, P.W. Sorensen and J.G. Dulka, unpublished results).

The finding that males exhibit circadian differences in endocrine sensitivity to the stimulus of PG-females, but not to 17,20B-P, suggests that these two stimuli act through separate neuroendocrine mechanisms to regulate GtH release in males. Although the reason for this difference is not known, it may not be surprising that males lack a marked day-night difference in endocrine responsiveness to water-borne 17,20B-P, considering the extended time period (22:00-06:00 h) over which females normally release 17,20B-P (Stacey et al., 1989). Since the release of 17,20B-P encompasses both the photophase and scotophases, selection pressures may not have operated to restrict male responsiveness to one of the time periods.

In Chapter 7, blood concentrations of GtH and GH were monitored in males exposed to preovulatory (17,20B-P-releasing) or postovulatory (PG-releasing) females which were treated with hCG to induce ovulation. Those females which underwent final oocyte maturation and/or eventually ovulated had increased blood levels of 17,20B-P and released

17,20B-P to the water in amounts sufficient to induce a GtH response in males (Fig. 7.1). These findings provide further evidence that the stimulatory action of 17,20B-P on males is primarily associated with the period of final oocyte maturation in females. In addition, the GtH response to water-borne 17,20B-P occurs in both grouped and isolated males (Chapter 7) and, in itself, does not appear to be influenced by social interaction with females (Stacey and Sorensen, 1986). Taken together, the results provide further evidence that water-borne 17,20B-P signifies impending ovulation and triggers reflex GtH responses which allow milt production to be synchronized with ovulation in goldfish.

In contrast, although males increase GtH following exposure to PG-injected or ovulated females, both of which release a PG-like spawning pheromone, the PG-pheromone alone has no effect on male GtH. For example, in the present study, individual males allowed direct access to ovulated females had increased GtH levels within 1 h of spawning (Chapter 7). Furthermore, males permitted to spawn with PG-injected females had GtH increases which were identical to those of males which spawned with ovulated females. However, individual males failed to exhibit a GtH response if they were exposed to detectable concentrations of water-borne PGFs (Sorensen et al., 1989a), or separated from ovulated females by a partition that restricted physical contact but allowed water exchange. Thus, although males become sexually aroused by the PG pheromone, they require behavioral interactions with PG-releasing females before GtH responses can be triggered.

These findings suggest that the PG and 17,20B-P pheromone systems operate through different neuroendocrine mechanisms to regulate GtH release in males. In support, water-borne 17,20B-P and PGs are detected by different olfactory receptors (Sorensen et al., 1987; Sorensen et al., 1988) which may give rise to distinct neuroendocrine pathways. Although both pathways are known to course centrally through the medial olfactory tracts (MOT; Stacey and Kyle, 1983; Chapter 3), there is considerable evidence that the two pathways diverge at the level of the forebrain. As discussed above, reflex GtH responses to 17,20B-P may involve the activation of a direct olfactory pathway to the POA, causing a

reduction of DA turnover in the pituitary (Chapter 8). In contrast, GtH responses to PG-releasing females do not appear to be reflexive in that they are dependent on the performance of socio-sexual behavior by the male. This suggests that GtH release in males is regulated, at least in part, by brain areas that control male socio-sexual behavior. Two areas of the ventral telencephalon, the *area ventralis telencephali pars supercommissuralis* (Vs) and the *area telencephali pars ventralis* (Vv), have been implicated in the control of male reproductive behavior in goldfish (Kyle et al., 1982; Kyle and Peter, 1892; Koyama et al., 1984). Both these areas receive olfactory projections from the MOT (Von Bartheld et al., 1984; Levine and Dethier, 1985; Springer, 1983). Because conspecific interactions appear necessary for PG-induced GtH increases (Sorensen et al., 1988), the Vv-Vs regions may relay electrical impulses to the POA once the male's socio-sexual behavior is initiated. It is proposed that a functional Vv-Vs- preoptic connection (through the medial forebrain bundle) may serve as the anatomical substrate by which the PG pheromone can indirectly influence GtH release by activating male sexual behavior (Fig. 7.6). Presumably, GtH release under these conditions would be regulated through mechanisms similar to those which regulate GtH release in response to water-borne 17,20B-P.

Unlike the effects of water-borne 17,20B-P or the performance of spawning behavior on male GtH release, changes in circulating concentrations of GH were not consistently observed in males exposed to the odor of 17,20B-P alone or that of preovulatory or postovulatory females. In addition, spawning interaction with PG-injected or postovulatory females had no effect on male GtH release. Thus, the 17,20B-P and PG pheromones do not appear to stimulate GH release directly, nor does the performance of spawning behavior influence GH release in males. The lack of a GH change in males examined under these conditions is interesting since GnRH has been shown to stimulate the release of both GtH and GH in male and female goldfish (Chang et al., 1982; Marchant et al., 1988). Thus, the lack of GH changes in response to pheromonal or behavioral stimuli might imply that GnRH is not involved in the regulation of GtH responses to water-borne 17,20B-P and/or spawning females. However, it is possible that the release of GtH and GH is regulated by separate populations of hypothalamic GnRH neurons, and only those neurons

which control GtH release might be activated following exposure to 17,20B-P or spawning females. Alternatively, exposure to 17,20B-P might affect GnRH release to the gonadotropes, but not the somatotropes, by reducing DA inhibition to those GnRH nerve terminals that only innervate gonadotropes (Chapter 8). However, although a DA mediated mechanism for GnRH release may account for differences in GtH and GH secretion in 17,20B-P-exposed males, the direct activation of GnRH release by pheromonal input cannot be ruled out.

The experiments described in Chapter 8 examined whether the male GtH response to water-borne 17,20B-P is mediated through the actions of GnRH and/or DA. Attempts at measuring changes in brain and PIT levels of DA alone failed to indicate a clear relationship between the possible involvement of this compound and GtH release in 17,20B-P-exposed males. However, alterations in DA turnover, as determined by measuring changes in the ratio of DOPAC to DA content in the PIT, provided a clear means of assessing the central action of water-borne 17,20B-P on GtH release. Water-borne 17,20B-P significantly reduced DA turnover in the PIT within 20 min of exposure to the pheromone. In addition, the 17,20B-P-induced reduction in DA turnover persisted for at least 2 h, the longest time after exposure that tissue samples were obtained. Since the reduction in DA turnover in the PIT is inversely correlated with periods of increased GtH release, the results strongly suggest that water-borne 17,20B-P increases male GtH at least by causing a rapid abatement of DA release to the PIT.

Changes in brain and PIT concentrations of sGnRH and cGnRH-II were not observed in males which were simply exposed to 17,20B-P, suggesting that these forms of GnRH are not involved in mediating the GtH response to the pheromone. However, experiments (Chapter 8) which examined the effects of DA synthesis blockers on the GtH response to 17,20B-P indicate that reduced DA turnover may enhance the production and/or release of GnRH to the PIT. For example, injections of CARB, which significantly reduced DA content in the PIT, also resulted in significantly higher levels of PIT GnRH and serum GtH in 17,20B-P-exposed males compared to saline-injected controls. These

results suggest that the GtH response to water-borne 17,20B-P may be regulated through the combined actions of DA and GnRH. In support, recent studies indicate that catecholamines modulate the release of GnRH in goldfish. For example, NA and A stimulate, whereas DA inhibits, GnRH neurons centrally, as well as at the level of GnRH terminals in the PIT (Peter et al., 1989). Thus, it is proposed that water-borne 17,20B-P causes an immediate reduction in DA turnover in the PIT which influences GtH both directly, and perhaps indirectly through modulation of GnRH release to the PIT.

A number of questions concerning the possible involvement of DA and GnRH in mediating GtH responses to water-borne 17,20B-P in male goldfish remain to be examined. As discussed above, there is a need to examine the precise time-course of the 17,20B-P-induced reduction in DA turnover in the PIT. Although rapid changes in DA turnover may account for the initiation of GtH release in pheromone-exposed males, it is unclear if 17,20B-P causes long term reductions in DA inhibition, or whether such changes are responsible for maintaining increased levels of GtH for extended periods of time (Chapter 5). It also is not known to what extent GnRH contributes to maintaining elevated levels of GtH in pheromone-exposed males; such studies may require the use of specific GnRH antagonists, or more sophisticated techniques for measuring changes in GnRH in goldfish. In addition, the specificity of neuroendocrine changes associated with 17,20B-P-exposure remain to be studied in more detail. For example, although 17,20B-P causes reductions in DA turnover, it is not known if the pheromone also affects other catecholamine systems (NA and 5HT) as well.

These proposed studies would be most valuable if they could be extended to other situations in which goldfish rapidly increase GtH. For example, does the performance of male sexual behavior lead to similar or different changes in DA turnover compared to that observed observed following exposure to water-borne 17,20B-P? This information would indicate whether these two forms of social stimulation act through different neuroendocrine mechanisms to increase GtH release in male goldfish (see: Chapter 7). Also, it would be interesting to examine whether the onset of the ovulatory GtH surge in females is regulated by an abrupt reduction in DA inhibition, or whether the surge is primarily triggered through

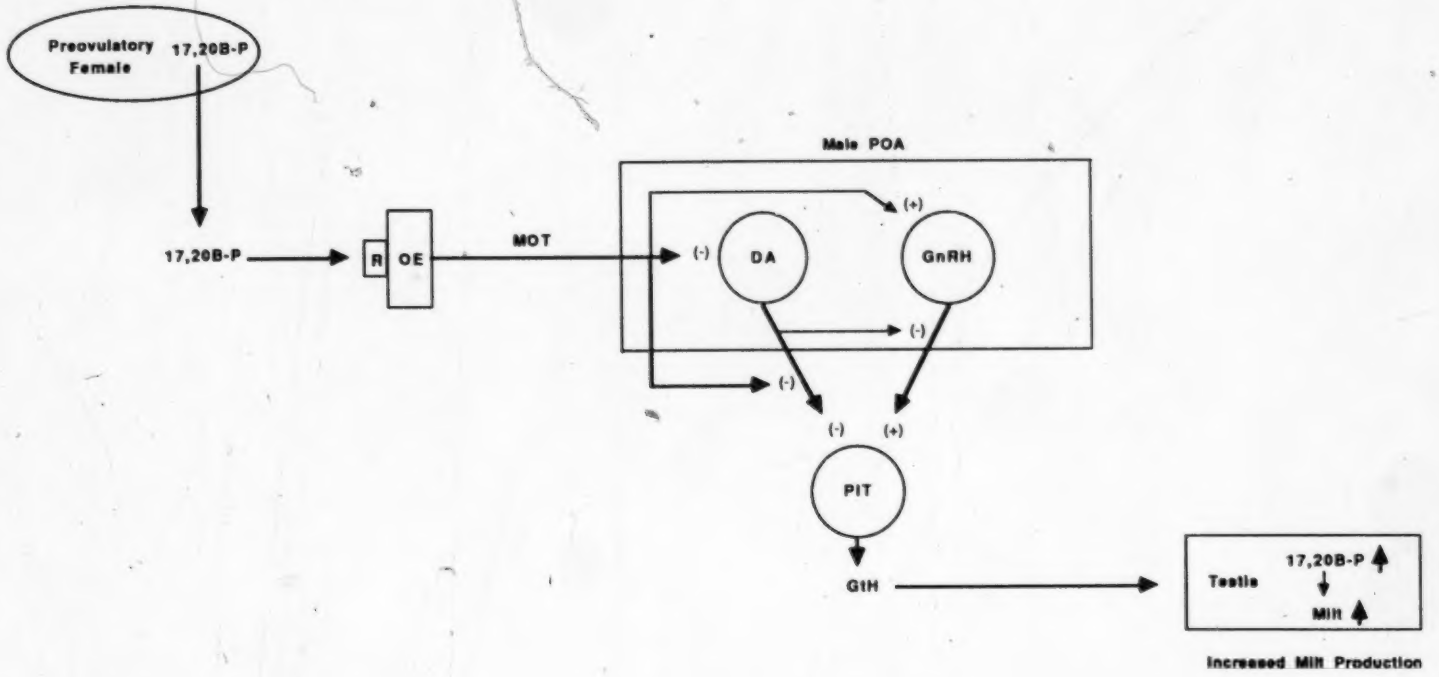
increased stimulation by GnRH, as suggested by other studies (Yu et al., 1988; Breton et al., 1988a). Answers to these questions may help explain why both GtH and GH increase simultaneously in the blood of ovulating female goldfish (Marchant, 1983) whereas only GtH increases in males exposed to water-borne 17,20B-P and PG-releasing females (Dulka et al., 1987b; Chapters 4 and 5). Could these differences be due to the possibility that GnRH plays a dominant role in regulating GtH and GH release in females while the inhibitory systems (i.e. DA for GtH; SRIF for GH) predominate in males? Hopefully these questions will provide directions for future research on the neuroendocrine regulation of GtH and GH secretion in goldfish and other teleosts.

On a broader scale, it is not known if other species of fish use 17,20B-P or 17,20B-P-like compounds as sex pheromones, although 17,20B-P has been implicated in the control of oocyte final maturation in a number of fresh water species (Nagahama et al., 1983; Goetz et al., 1983). However, the use of gonadal steroids and their metabolites as sex pheromones may be widespread in teleosts. For example, the 5B-reduced androgens, etiocholanolone glucuronide and 5B-pregnane-3α,17α-diol-20-one glucuronide, have been proposed to function as behavioral-releasing pheromones in the black goby (Colombo et al., 1982) and African catfish (Resink et al., 1988), respectively. Theories associated with the possibility that fish have evolved the use of hormones and their metabolites as pheromones have been discussed in detail by others (Doving, 1976; Colombo et al., 1982; Sorensen and Stacey, 1989). Briefly, 1) hormones and their metabolites represent pre-existing signals produced in temporal synchrony with discrete reproductive events, 2) they are readily excreted to the water, and 3) preexisting internal endocrine receptors may have been externalized to function as olfactory receptors for water-borne hormones. In addition, species-specificity of pheromone use in teleosts may be achieved by using different mixtures of hormones, or by modifying hormonal metabolic pathways for the production of hormonal pheromones (Sorensen and Stacey, 1989).

In conclusion, the major findings of this thesis have been incorporated into a model of the neuroendocrine regulation of the GtH and milt responses to water-borne 17,20B-P in male goldfish (Fig 9.1). Under appropriate environmental conditions, preovulatory female

goldfish exhibit an ovulatory surge in GtH which induces ovulation 10-12 h later. Approximately 10 h prior to ovulation, female goldfish synthesize increased amounts of 17,20B-P which induce final maturation of developing oocytes. 17,20B-P is then released to the water where it functions as a potent olfactory stimulant in males. Specific olfactory receptors detect water-borne 17,20B-P and give rise to a central pathway which courses through the MOTs to innervate regions of the POA that regulate GtH release in males. The POA contains both DA and GnRH immunoreactive neurons which project to the PIT. Water-borne 17,20B-P evokes increased GtH secretion in males by causing a rapid reduction in DA turnover in the PIT; however, reduced DA inhibition to the PIT may also allow GnRH to stimulate GtH release. Males chronically exposed to water-borne 17,20B-P have elevated levels of GtH for at least 8-12 h. During the first few minutes of exposure, the elevated levels of GtH stimulate the production of testicular 17,20B-P which promotes increased milt production within 4-6 h. Both the temporal pattern of 17,20B-P release from preovulatory females and the latency of the GtH and milt responses in males suggest that 17,20B-P functions as a sex pheromone which synchronizes milt production with ovulation in goldfish. Intense male-male competition for access to ovulated females is believed to be the primary factor which has lead to the evolution of the pheromone system. This may be the only vertebrate system in which the identity, source and release of the pheromone, as well as the sensory pathway, neuroendocrine-endocrine mediators and the possible significance of the final response in the pheromone recipient, all are clearly established. In this regard, the 17,20B-P pheromone system in goldfish serves as an excellent comparative model to study the regulation of vertebrate endocrine responses to sex pheromones in general.

Figure 9.1. Proposed model of the 17,20B-P pheromone system in goldfish. Preovulatory females release increased amounts of 17,20B-P to the water approximately 10 h prior to ovulation. Specific receptors (R) on the olfactory epithelium (OE) are involved in the detection of water-borne 17,20B-P by males. These receptors give rise to a central pathway which courses through the medial olfactory tracts (MOT) to reach the preoptic area (POA) in the ventral hypothalamus. Water-borne 17,20B-P stimulates reflex gonadotropin (GtH) release in males by a rapid reduction in dopamine (DA) turnover in the pituitary (PIT), presumably by altering the activity of DA neurons in the POA. Reduced DA inhibition may also stimulate gonadotropin-releasing hormone (GnRH) release to the PIT and enhance GtH release. Elevated levels of serum GtH stimulate rapid production of 17,20B-P by the testis. Increased levels of testicular 17,20B-P are thought to mediate the action of GtH on milt production. Because males are capable of increasing milt volume within 4-6 h of exposure to water-borne 17,20B-P, it is proposed that this pheromone system functions to synchronize milt production with ovulation in goldfish. (-) and (+) indicate inhibition and stimulation, respectively. See text for further details.


Preovulatory Female
17,20B-P
17,20B-P
R
OE
MOT
Male POA
(-)
DA
GnRH
(+)
(-)
(-)
(-)
(+)
PIT
GtH
Testis
17,20B-P
Milt
Increased Milt Production

9.1 LITERATURE CITED

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10. APPENDIX I

Dulka, J.G., N.E. Stacey, P.W. Sorensen, and G.J. Van Der Kraak. 1987. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. Nature 325: 251-253.

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A steroid sex pheromone synchronizes male–female spawning readiness in goldfish

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Understanding of pheromone function in teleost fish has been impeded by a lack of information on pheromone identities[1]. Our recent studies[2,3] on goldfish *Carassius auratus*, however, provide strong evidence that 17α,20β-dihydroxy-4-pregnen-3-one (17,20P), the proposed oocyte maturation-inducing steroid hormone in goldfish[4] and other teleosts[5], could be a potent female sex pheromone. Milt (sperm and seminal fluid) volume in goldfish is increased by exposure to 17,20P (and to a lesser extent by exposure to two precursors of 17,20P, progesterone and 17α-hydroxyprogesterone) but not to other steroids proposed as fish pheromones[1,6,7]. In addition, the goldfish olfactory epithelium is extremely sensitive to 17,20P (ref. 3), and the increase in milt volume normally induced by 17,20P exposure is abolished by sectioning the medial olfactory tracts, which previously have been implicated in the control of sex behaviour in male goldfish[8]. We report here that ovulating goldfish release 17,20P into the water and that a rapid (within 15 min) elevation in blood gonadotropin of males mediates the milt response to 17,20P exposure. We conclude that this pheromone system synchronizes milt production with ovulation.

The goldfish is well suited for studies of pheromone release by females and pheromonal effects on males because ovulation is synchronized with the light-dark cycle and can be manipulated under laboratory conditions. Goldfish which have completed vitellogenesis at 12–14 °C ovulate spontaneously within a day of being warmed to 20 °C and exposed to aquatic vegetation, the substrate for oviposition. On a 16-hours light : 8 hours dark photoperiod, the preovulatory gonadotropin (GtH) surge begins after the midpoint of the photophase and induces ovulation (follicular rupture) about 10–12 hours later, during the latter half of the scotophase[9]; oocyte final maturation is completed halfway between the initiation of the GtH increase and ovulation[10]. Spawning begins shortly after ovulation.

To determine whether or not females release 17,20P, six mature females were maintained individually in 1 litre of water for four successive four-hour intervals encompassing the periovulatory period. Three of the six females ovulated during the third four-hour interval and released high levels of 17,20P into the water during the first and second four-hour intervals (Fig. 1). All water samples from the three females which did not ovulate had relatively low concentrations of 17,20P.

We next used hypophysectomized males to determine whether exposure to 17,20P increases milt volume by stimulating the pituitary-gonadal axis. When hypophysectomized males were stripped of milt[11] two days after surgery and then exposed to a water concentration of 17,20P (5×10^{-10} M) approximating the peak concentrations released by ovulating females (Fig. 1), milt volumes which could be stripped the following morning were significantly ($P < 0.001$) reduced. In contrast, milt volumes of males which had undergone a sham operation and of intact males were significantly ($P < 0.001$) increased following this exposure to 17,20P (Fig. 2).

We determined whether GtH, known to increase milt production when injected into a variety of teleosts including goldfish[12], increases in the blood of males exposed to water-borne 17,20P. We also measured serum 17,20P levels as 17,20P of testicular origin has been shown to mediate the action of GtH on milt production[13]. Experimental males exposed during the scotophase to 17,20P (5×10^{-10} M) for 15, 30, 60, or 120 min

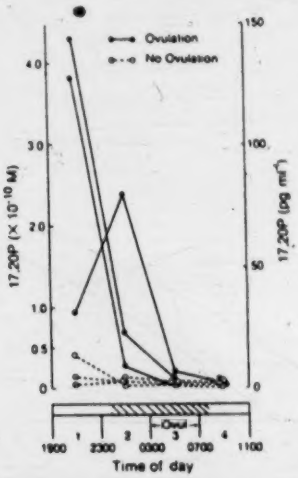

Fig. 1 Concentrations of 17,20P in female holding water during the periovulatory period.
Methods. At 21:00 h on day 1, six mature female goldfish (Ozark Fisheries, Stoutland, Missouri) were transferred from stock tanks (14 °C; 16L:8D photoperiod, lights on at 08:00 h) to aquaria (20 °C) containing aquatic vegetation. At 19:00 h on day 2, when the preovulatory surge of GtH (ref. 9) should have commenced, females were removed and placed individually in glass jars containing 1 litre of aerated water at 20 °C. At 23:00 h, a 10 ml water sample was taken from each jar for 17,20P determination and each female was transferred to a clean jar. This procedure was repeated four times, the last water sample being taken at 11:00 h on day 3. Three of the six females ovulated, all during the third sample period (03:00–07:00 h). Water samples were extracted two times with 2 vol ether and the amount of 17,20P in the extract determined by radioimmunoassay[15,16].

had significantly ($P < 0.01$) higher blood GtH concentrations than did control males exposed to the ethanol vehicle for 60 min (Fig. 3*a*). As well, milt volumes stripped 7–8 h after 17,20P exposure were significantly ($P < 0.01$) greater in the experimental groups than in the control group (Fig. 3*c*). Consistent with the hypothesis that endogenous 17,20P mediates GtH-induced milt increase in goldfish, serum 17,20P levels in three of the four groups exposed to water-borne 17,20P were significantly ($P < 0.01$) higher than in control males exposed to ethanol (Fig. 3*b*). It is unlikely that passive uptake of 17,20P from the aquarium water accounts for these differences between experimental and control groups because serum 17,20P levels in all groups were higher than the calculated water concentration of 17,20P.

A pheromone-mediated increase in milt volume could affect reproductive success if the increase occurs prior to ovulation and spawning. We therefore determined the latency of the 17,20P-induced milt response. Males exposed to 5×10^{-10} M 17,20P or ethanol at the onset of the scotophase were anaesthetized either three or six hours later for blood sampling and determination of milt volume. At both sample times, serum GtH levels in males exposed to water-borne 17,20P were significantly higher ($P < 0.01$ and $P < 0.05$, respectively) than those of control males (Fig. 4*a*). However, only at the 6 h sample time were the milt volumes of males exposed to 17,20P greater ($P < 0.05$) than those of controls (Fig. 4*b*). As peak preovulatory release of 17,20P from females occurs more than 4 h prior to ovulation (Fig. 1), the milt response to water-borne 17,20P appears to be rapid enough to allow an increase in milt volume prior to spawning.

The results presented in this and our earlier studies[2,3] clearly show that 17,20P, which promotes oocyte final maturation in

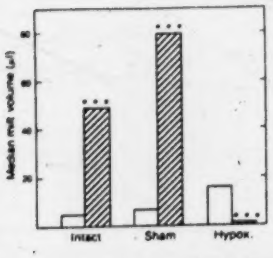

Fig. 2 Effect of hypophysectomy on the 17,20P-induced increase in milt volume in male goldfish. Open bars, initial milt volume; shaded bars, milt volume after exposure to 17,20P. Males were hypophysectomized (Hypox.), had undergone a sham operation (Sham) or were intact (Intact); ***, significantly different from pre-exposure values ($P < 0.001$); $n = 20$ in all groups.
Methods. As in other experiments (Figs 3 and 4), mature male goldfish were maintained before use in flow-through stock tanks at 20 °C on a 16L:8D photoperiod (lights on at 08:00 h). On day 1, males were anaesthetized (0.1% 2-phenoxyethanol, Syndel), then either hypophysectomized by the opercular approach[17], sham operated, or handled without surgery, and placed by groups in 65 l flow-through aquaria (four fish per aquarium) at 20 °C. At 17:00 h on day 3, fish were anaesthetized and stripped of milt to determine initial milt volumes. The fish were placed belly-up in a slotted foam pad and gentle finger pressure was applied to the abdomen to express milt into weighed (±0.1 mg) haematocrit tubes with weighed caps which were then weighed to determine the weight of milt. Milt density was assumed to be 1.0 and milt values are expressed as volumes (μl) rather than as weights. At 21:00 h, water flow to all aquaria was shut off and 10 μg 17,20P (Sigma) in 0.1 ml of ethanol was added to each aquarium; this dose should have produced a water concentration of ~150 pg ml^{-1} (5×10^{-10} M), although actual water concentrations were not measured. At 09:00 h on day 4, all fish were again anaesthetized and stripped to determine milt volumes following exposure to 17,20P. Within each group, milt volumes before and after 17,20P exposure were compared by the Wilcoxon matched-pairs signed ranks test.

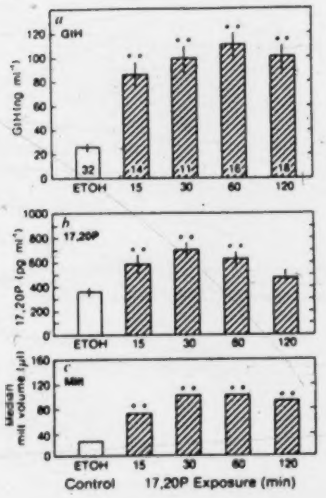

Fig. 3 Effect of water-borne 17,20P on serum GtH (*a*), serum 17,20P (*b*), and (*c*) milt volume in male goldfish. Values in *a* and *b* are means; lines, ±s.e.m. range; **, significantly different from controls ($P < 0.01$). Numbers within bars in *a* show sample size.
Methods. Mature males were placed in 65 l flow-through aquaria (four fish per aquarium) at 20 °C. During scotophase, 10 μg of 17,20P in 0.1 ml of ethanol was added to each experimental aquarium and blood samples were taken from different groups of males under anaesthesia 15, 30, 60, and 120 min later; control males were bled following 60 min exposure to ethanol vehicle. All blood samples were taken during the scotophase (02:00–06:00 h) and all treatment groups were balanced over time. Following blood sampling, all fish were transferred to clean flow-through aquaria and milt sampled 7–8 h after exposure to 17.20P had begun. Serum GtH (ref. 18) and 17,20P (refs 15,16) concentrations were determined by radioimmunoassay. Data on GtH and 17,20P were analysed by ANOVA and Newman-Keuls procedure. Milt volume data were analysed by Kruskal-Wallis ANOVA and Mann-Whitney *U* tests.

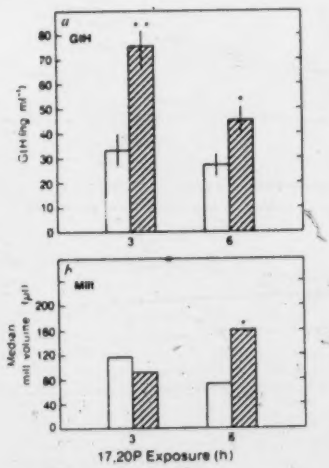

Fig. 4 Effect of 3 and 6 hours exposure to water-borne 17,20P on serum GtH (*a*) and milt volume (*b*) in male goldfish. Values in *a* are means; lines, ±s.e.m. range; **, $P < 0.01$; *, $P < 0.05$ (significantly different from controls); $n = 15$ in all groups.
Methods. Mature males were placed in 65 l aquaria (three fish per aquarium) at 20 °C. At the onset of scotophase, either 0.1 ml ethanol (open bars) or 10 μg of 17,20P in 0.1 ml of ethanol was added to each aquarium. Three and 6 h later, different groups of males were anaesthetized for blood sampling and determination of milt volume. Data on GtH were analysed by *t*-tests and milt volume data were analysed by Mann-Whitney *U* tests.

goldfish[4] and is released from females prior to ovulation, also increases blood GtH and milt volume in males. Indirect evidence that these physiological responses of males are normally stimulated by females is provided by the recent observation[14] that the presence of an ovulating female goldfish induces a GtH surge in males coincident with that of the female. Both the temporal pattern of 17,20P release from preovulatory females and the latency of the milt response to water-borne 17,20P support our proposal that 17,20P is a pheromone which synchronizes milt production with ovulation. This appears to be the only vertebrate system in which the identity, cellular source and release of the pheromone, as well as the sensory pathway, endocrine mediators and significance of the response in the pheromone recipient, all are clearly established.

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11. APPENDIX II

Stacey, N.E., P.W. Sorensen, G.J. Van Der Kraak, and J.G. Dulka. 1989. Direct evidence that 17o ,20B-dihydroxy-4-pregnen-3-one functions as a goldfish primer sex pheromone: preovulatory release is closely associated with male endocrine responses. Gen. Comp. Endocrinol. 75: 62-70.

GENERAL AND COMPARATIVE ENDOCRINOLOGY 75, 62–70 (1989)

Direct Evidence that 17α,20β-Dihydroxy-4-pregnen-3-one Functions as a Goldfish Primer Pheromone: Preovulatory Release Is Closely Associated with Male Endocrine Responses

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This study directly tested the hypothesis that 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) is a goldfish preovulatory pheromone (pheromone released at peak levels during oocyte final maturation) which increases blood gonadotropin (GtH) and milt volume in males. During spontaneous ovulation, GtH and 17,20β-P in female blood and 17,20β-P released to the water increased dramatically 7–10 hr prior to ovulation, peaked 1–4 hr prior to ovulation, and then rapidly declined. Males held with these females, or exposed to their odors, had increased GtH levels and milt volumes at approximately the time when increased 17,20β-P release by ovulatory females commenced. Although these findings strongly support the hypothesis that 17,20β-P is a preovulatory female sex pheromone in goldfish which stimulates male GtH levels and milt production prior to spawning, the milt increases occurred earlier than predicted, suggesting either that preovulatory 17,20β-P release begins earlier than the data indicate or that other steroids known to have pheromonal activity are released before 17,20β-P. © 1989 Academic Press, Inc.

Recent studies have demonstrated that, shortly before ovulating, female goldfish release a pheromone which has rapid effects on the male reproductive endocrine system. Kobayashi *et al.* (1986a) found that if a male goldfish is placed with a female undergoing spontaneous ovulation (ovulatory female), the male's circulating gonadotropin (GtH) rises in synchrony with the female's periovulatory GtH surge. Because this GtH increase in the male is abolished if the olfactory tracts are sectioned, but is unaffected if the sexes are separated by an opaque barrier allowing water flow, Kobayashi *et al.* (1986b) hypothesized that the male's endocrine response is triggered by a female pheromone. Our studies (Stacey and Sorensen, 1986; Dulka *et al.*, 1987; Sorensen *et al.*, 1987) suggest that this preovulatory pheromone is 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), the proposed oocyte maturation-inducing steroid in goldfish (Nagahama *et al.*, 1983).

Although no study has yet demonstrated that, in groups of goldfish, male GtH increases in synchrony with the release of 17,20β-P by ovulatory females, we have clearly shown that isolated females release greatly increased quantities of 17,20β-P shortly before ovulating (Dulka *et al.*, 1987; Van Der Kraak *et al.*, 1989). Additionally, we have found that if males are exposed to low (100 p*M*) concentrations of 17,20β-P, their GtH increases within 15 min and their milt (sperm and seminal fluid) volume increases within 6 hr (Dulka *et al.*, 1987). Finally, lesioning experiments have shown that the endocrine and testicular responses to water-borne 17,20β-P are mediated by the olfactory system (Stacey and Sorensen, 1986 and unpublished), and extracellular

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electrical recording (electro-olfactogram; EOG) has demonstrated that the goldfish olfactory epithelium is extremely and specifically sensitive to this steroid (Sorensen *et al.*, 1987 and unpublished).

We have tested the pheromonal activity of more than 30 steroids using three bioassays (EOG, GtH increase, milt volume increase) and found that pheromonal activity is restricted to C21 progestational steroids, the most potent of which is 17,20β-P. Although glucuronated androgens and estrogens have been proposed as sex pheromones in some fish (Colombo *et al.*, 1982; Van Den Hurk and Lambert, 1983), these conjugated steroids have no activity in our three pheromone bioassays (Stacey and Sorensen, 1986; Sorensen *et al.*, 1987). Recently, Resink *et al.* (1987b) have shown that a glucuronated, A-ring-reduced metabolite of 17α-hydroxyprogesterone (17P) is a potent olfactory stimulant in the African catfish (*Clarias gariepinus*), and have suggested that this steroid is a component of the pheromone from seminal vesicles which attracts ovulated females (Resink *et al.*, 1987a, b). Unfortunately, we have been unable to determine whether 17,20β-P-glucuronide (17,20β-P-G), a metabolite of 17,20β-P which is released by preovulatory goldfish (Van Der Kraak *et al.*, 1989), has pheromonal activity because it is not commercially available.

This study sought to test directly whether 17,20β-P could be functioning as the preovulatory pheromone of goldfish by simultaneously measuring the periovulatory profile of 17,20β-P released by female goldfish in conjunction with the blood GtH levels and milt volumes of males exposed to their odors. Males were maintained both with females ("contact") and physically isolated from them but in their water ("noncontact") to determine the contribution of the pheromone to changes in male GtH and milt. Female blood samples were assayed as well to determine periovulatory profiles of circulating GtH and 17,20β-P, and 17,20β-P-G was measured in female water samples to determine whether the profile of release of this conjugated steroid was correlated with changes in male GtH levels and milt volumes.

MATERIALS AND METHODS

Animals. Goldfish (15–45 g) of the common or comet variety were purchased from Ozark Fisheries Co., Stoutland, Missouri in March 1987, separated by sex, and held in 1000-liter flow-through stock aquaria at 14–15° under a 16L:8D photoperiod (lights on at 0800 hr). Fish were fed with Oregon Moist trout pellets or Nutrafin flaked food *ad libitum* at least once daily.

Ovulation aquaria. Seventy-liter opaque glass aquaria maintained under the same photoperiod as stock aquaria were set on water tables which were tilted along the long axis of the aquaria so that water entered at one end and overflowed from the other (Fig. 1). An opaque plastic screen barrier divided each aquarium transversely but allowed water exchange between the two compartments. Each aquarium was provided with gravel substrate, an aeration stone in the overflow compartment, and a generous supply of floating artificial vegetation to promote spontaneous ovulation (Stacey *et al.*, 1979a, b).

Protocol. Spontaneous ovulation in goldfish is synchronized with photoperiod and occurs readily under controlled laboratory conditions (Stacey *et al.*, 1979a, b). When mature female goldfish are held on a 16L:8D photoperiod, warmed from cool (14–15°) water to 20°, and exposed to aquatic vegetation (spawning substrate), many individuals commence a spontaneous preovulatory GtH surge near midphotophase of the following day. Most ovulations (defined as the time when mature oocytes can first be expressed from the ovipore by gentle pressure on the abdomen) occur in the latter half of the following scotophase (Stacey *et al.*, 1979a, b). This study took advantage of the tempo-

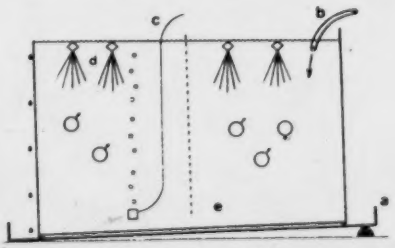

FIG. 1. Ovulation aquarium. (a) Tilted water table. (b) Water inlet. (c) Air supply. (d) Artificial vegetation. (e) Gravel substrate.

ral synchrony of ovulation among females to determine periovulatory profiles of GtH and 17,20β-P in female blood, 17,20β-P released to the water, and GtH levels and milt volumes in males exposed to females.

Fish were removed from stock aquaria at 2200 hr on Day 1 and placed in ovulation aquaria at 14–15°. Five fish were placed into each ovulation aquarium, two contact males and one female in the inflow compartment and two noncontact males in the outflow compartment. The temperature of the incoming water was then increased so that the aquaria gradually warmed to 20–21° by the onset of photophase on Day 2.

To obtain blood and water samples during the preovulatory period, fish in groups A–E were sampled at 3-hr intervals from 1400 hr on Day 2 (expected to be prior to the onset of the GtH surge) until 0200 hr on Day 3 (expected to be prior to the occurrence of the first ovulations) (Fig. 2). Group F was sampled at 0600 hr on Day 3 to provide samples from males and recently ovulated females which (as with the preovulatory fish in groups A–E) were undisturbed prior to sampling. All fish were removed from an aquarium at the same time, a dim red flashlight being used for this purpose during scotophase. Males were separated from the female, anesthetized in 2-phenoxyethanol (Syndel; 0.05%), a blood sample taken from the caudal vasculature, and milt stripped into preweighed capillary tubes for volume determination (Stacey and Sorensen, 1986). The female was then placed in a 3-liter glass jar containing 1.5 liter of aerated water (20°) and surrounded by an opaque barrier. Two hours later, 10-ml water samples for 17,20β-P and 17,20β-P-G determination were collected and frozen on dry ice in siliconized glass scintillation vials. The female was then anesthetized for blood sampling, returned to the ovulation aquarium (no males present), and checked for ovulation (without anesthesia) at 90-min intervals from 0330 to 0930 hr on Day 3. Female blood samples were taken after water sample collection to eliminate the possibility that pheromone release was influenced by anesthesia and bleeding.

The experiment was repeated three times (May 6–16, 1987), the first two runs with four ovulation aquaria and the third with five ovulation aquaria, at each of the six sampling times. Data from all three runs were combined for analysis.

Hormone assays. GtH levels in male and female blood and 17,20β-P levels in female blood were determined by radioimmunoassay (RIA) as described previously (Peter *et al.*, 1984; Scott *et al.*, 1982; Van Der Kraak *et al.*, 1984; Pankhurst *et al.*, 1986). 17,20β-P and 17,20β-P-G were extracted from female water samples and measured by RIA as described by Van Der Kraak *et al.* (1989). Water samples from only two nonovulatory females were measured for each of the sample times.

Data analysis. Because of variability in the timing of ovulations, males and females were reassigned to a new set of groups based on a standard time of ovulation rather than on time of day. To accomplish this, females which ovulated by 0630 hr on Day 3 were assigned an ovulation time of 0500 hr, and those which ovulated between 0630 and 0930 hr on Day 3 were assigned an ovulation time of 0800 hr. Both of these assigned ovulation times were then considered to be time zero (0600 hr), so that times of blood and water sample collections could be expressed in hours preceding and following the standardized ovulation time (0600 hr).

For example, the five females of group C which ovulated by 0630 hr on Day 3 were considered to have been placed in water sample jars 9 hr prior to ovula-

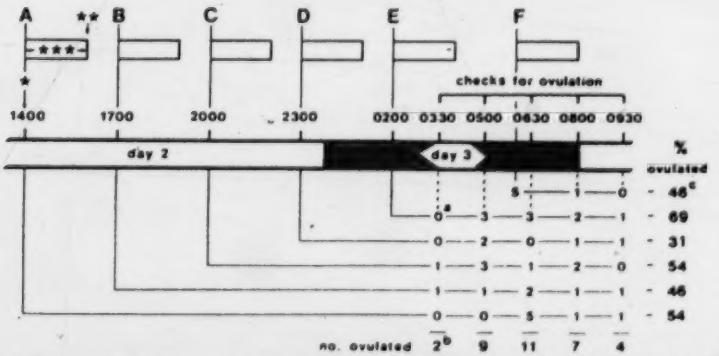

FIG. 2. Treatment schedules and ovulatory responses of the six experimental groups (A–F). Times of blood samples from males (*) and females (**) and water samples from females (***) are indicated for group A. Ovulatory response is shown as the number of females of each group which ovulated at each ovulation check[a], the total number of females in groups A–E which ovulated at each ovulation check[b], and the percentage of females in each group (n = 13) which ovulated in the experiment[c].

tion and to have been blood sampled 7 hr prior to ovulation (Fig. 2). Data from these five group C females and the "contact–ovulatory" and "noncontact–ovulatory" males in their aquaria were pooled with data from the two group D females which ovulated between 0630 and 0930 hr and were compared with data from the six group C females which failed to ovulate and the nonovulatory males in their aquaria. Data from the two group C females which ovulated between 0630 and 0930 hr were pooled with data from the four group B females which ovulated by 0630 hr and were compared with the group B females which failed to ovulate. The five group F females which were ovulated when first checked at 0600 hr on Day 3 were assigned an ovulation time of 0600 hr and a blood sample time of 2 hr postovulation.

Female hormone data were analyzed following log transformation; differences among the six periovulatory sample times were analyzed separately for ovulatory and nonovulatory females using one-way ANOVAs and Student–Neuman–Keuls followup tests. For each sample time, differences between blood hormone levels of ovulatory and nonovulatory females were analyzed by *t* test.

Male GtH data were log-transformed and then analyzed using ANOVA and Student–Neuman–Keuls tests to compare changes over time within each of the four male groups (noncontact–nonovulatory, contact–nonovulatory, contact–nonovulatory, and contact–ovulatory). For each sample time, ANOVA and Student–Neuman–Keuls tests were used to compare data from the four male groups. Milt volume data were not normally distributed. Accordingly, differences in milt volume at each of the six sample times were compared by using Kruskall–Wallis ANOVA and an analog of Tukey's test (Biostat I; Sigmasoft, Placentia, CA). The noncontact–nonovulatory and noncontact–ovulatory groups were compared with the Mann–Whitney *U* test to assess the effects of pheromone exposure alone.

RESULTS

Ovulation. Thirty-nine (50%) of the females used in this study ovulated (Fig. 2). As in our earlier studies (Stacey *et al.*, 1979a, b), the majority of ovulations occurred in the latter part of scotophase. There was no indication that the time at which females were placed in water sample jars affected either the proportion of females ovulating or the time at which ovulations occurred.

Female blood hormones. The periovulatory GtH surge commenced 13–10 hr prior to ovulation, reached peak levels between 4 and 1 hr prior to ovulation, and had begun to decline 2 hr after ovulation (Fig. 3A). GtH levels of nonovulatory females did not change throughout the sampling period and were significantly lower than those of ovulatory females at all but the initial sample time.

Blood 17,20β-P levels of nonovulatory females also did not change throughout the sampling period and were significantly lower than those of ovulatory females at all sample times. The periovulatory profile of blood 17,20β-P in ovulatory females was similar to that of GtH. A surge of 17,20β-P commenced approximately 3 hr after the GtH surge and declined more rapidly in the early postovulatory period; however, blood 17,20β-P levels in females sampled 2 hr after ovulation were still significantly higher than those in females sampled prior to the onset of the surge.

Steroid release. Water samples from nonovulatory females had uniformly low levels of 17,20β-P and 17,20β-P-G (4–12 and 15–34 ng/female, respectively) which showed no apparent pattern of change over the sampling period (Fig. 3B); similarly, low levels of 17,20β-P and 17,20β-P-G were measured in water from ovulatory females sampled 13 and 10 hr prior to ovulation. The release of 17,20β-P and 17,20β-P-G by ovulatory females was greater than at the first time period by 7 hr prior to ovulation, reached peak levels just prior to ovulation, and then dramatically declined. The release of 17,20β-P by ovulatory females 2 hr after ovulation was less than prior to the onset of the surge (despite the fact that blood levels at this time were significantly higher than presurge levels), whereas release of 17,20β-P-G was similar to presurge levels. Nonovulatory fish released more 17,20β-P-G than 17,20β-P throughout the sampling period. In ovulatory fish, 17,20β-P-G release predominated prior to and following the surge, whereas 17,20β-P release predominated at the peak of the surge (Table 1).

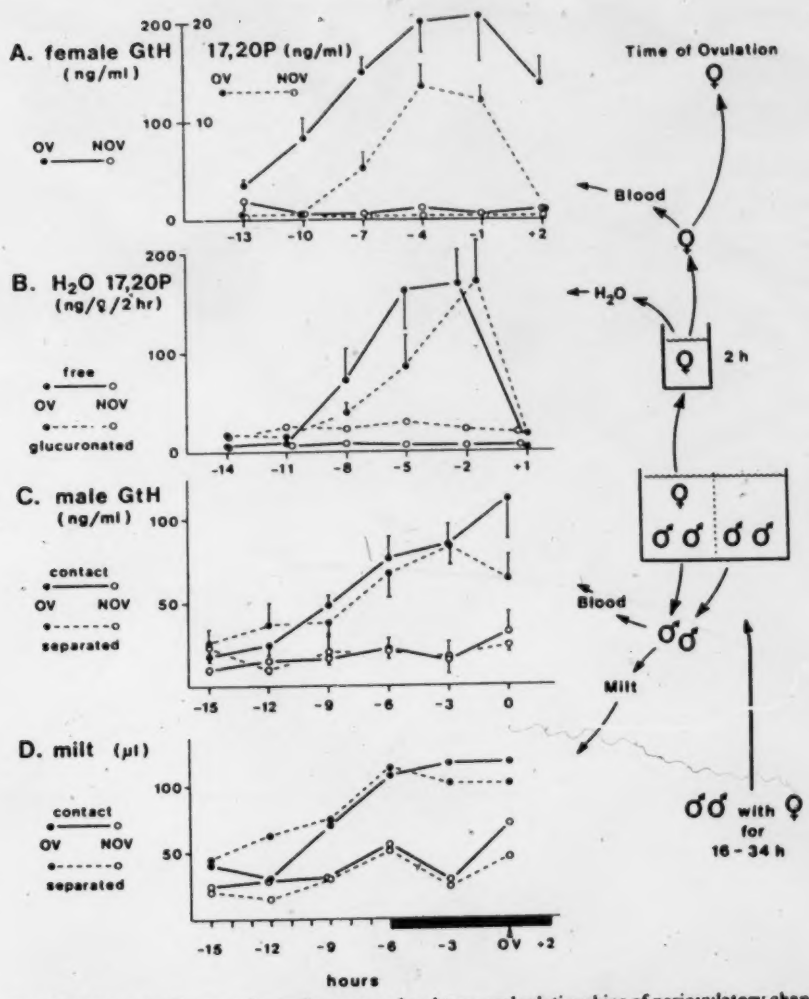

FIG. 3. Schematic diagram of sampling protocol and temporal relationships of periovulatory changes in male and female hormones. (A) Female blood GtH and 17,20β-P (±SEM). (B) Water levels of free and glucuronated 17,20β-P (±SEM). Data are plotted at the midpoint of the 2-hr water collection period. (C) Male GtH (±SEM). (D) Milt volume (median).

Male GtH. GtH levels of males placed with females which failed to ovulate (nonovulatory males) did not change during the sampling period and were not affected by whether the male was in contact with the female (Fig. 3C). In contrast, GtH levels of males placed with ovulatory females increased steadily during the preovulatory period. Contact–ovulatory males had GtH levels similar to those of the noncontact–ovulatory males at all but the postovulatory sample, when the levels of the noncontact males declined. Although GtH levels of ovulatory and nonovulatory males were not different 15 hr prior to ovulation, levels in the noncontact–ovulatory group were sig-

TABLE 1
RATIO OF FREE TO GLUCURONATED 17,20β-P IN WATER SAMPLES FROM NONOVULATORY AND OVULATORY FEMALE GOLDFISH

	n	Free/Gluc (SEM)
Nonovulatory	11	0.33 (+0.04)
Ovulatory		
−14 hr	7	0.44 (+0.09)
−11 hr	6	0.73 (+0.22)
−8 hr	7	1.48 (+0.32)*
−5 hr	5	2.72 (+1.03)*
−2 hr	7	1.37 (+0.39)
+1 hr	5	0.33 (+0.11)

* $P < 0.05$ vs −14 hr.

nificantly higher than the nonovulatory groups 12 hr prior to ovulation. After this time, GtH levels of both groups of ovulatory males were significantly greater than those of the nonovulatory male groups for the remainder of the periovulatory period.

Milt volumes. Milt volumes of ovulatory males were variable and higher than those of nonovulatory males, although differences among all four male groups were significant only 9 and 3 hr prior to ovulation (Fig. 3D). However, when only the noncontact groups (exposed to pheromones only) were compared, milt volumes of ovulatory males were greater than those of nonovulatory males at all but the first sample time.

DISCUSSION

This study strongly supports our hypothesis (Stacey and Sorensen, 1986; Dulka *et al.*, 1987; Sorensen *et al.*, 1987, 1988a, b) that 17,20β-P functions as a female preovulatory primer pheromone in goldfish. The clear temporal correlation between increasing 17,20β-P release by preovulatory females and increasing GtH levels and milt volumes in males exposed to their odors strongly suggests that the 17,20β-P pheromone plays an important role in stimulating milt production immediately prior to spawning. Additionally, the similar GtH and milt levels of males in contact with and separated from ovulatory females (with the exception of the postovulatory sample) indicate that water-borne pheromones are entirely responsible for the observed male endocrine changes.

The periovulatory profiles of blood GtH and 17,20β-P in this study are similar to those previously described in female goldfish by Kobayashi *et al.* (1987). This study indicates that the clearance of 17,20β-P from the blood to the water is surprisingly rapid, such that the profile of 17,20β-P release accurately reflects the progress of oocyte final maturation. Because it is not known whether any circulating 17,20β-P is metabolized to forms not detected by our 17,20β-P assay prior to release, the measures taken in this study do not permit a true estimate of 17,20β-P production and release. However, even if one conservatively assumes that all blood 17,20β-P is released without metabolism, a 20-g goldfish at the peak of its 17,20β-P surge (and having approximately 11 ng of total circulating 17,20β-P) would have to produce and release seven times this amount per hour to generate the water concentrations of 17,20β-P measured in this study.

The periovulatory profile of 17,20β-P release in this study is clearly different from that described in our earlier studies (Dulka *et al.*, 1987; Van Der Kraak *et al.*, 1989), in which release of 17,20β-P, 17,20β-P-G, and 17P all decreased prior to ovulation. Although the reason for this discrepancy is not known, we suspect that our earlier procedure for collecting water samples (in which females were held for four successive 4-hr intervals in only 1 liter of water) was stressful and may have depressed 17,20β-P synthesis by prematurely terminating the ovulatory GtH surge. Unfortunately, this cannot be confirmed, as no blood samples were taken in the earlier studies. If blood GtH and 17,20β-P were in fact depressed by this purportedly stressful sampling procedure (Dulka *et al.*, 1987;

Van Der Kraak *et al.*, 1989), then the fact that ovulations did occur indicates that ovulation *per se* does not require the high 17,20β-P levels normally seen in the latter half of the ovulatory GtH surge. It is not known whether these high levels of 17,20β-P synthesis and release are related to pheromonal function.

The low levels of 17,20β-P released by postovulatory females and the decreasing GtH levels of noncontact males exposed to their water indicate that water-borne 17,20β-P is not responsible for the high GtH levels of those males in contact with ovulated females. Rather, the high GtH levels of these males are thought to result from the combined effects of exposure to postovulatory prostaglandin pheromone and interaction with sexually receptive females (Kyle *et al.*, 1985; Sorensen *et al.*, 1988b). This interpretation differs from that of Kobayashi *et al.* (1986b) who concluded that a female goldfish ovulatory pheromone exerts both a "primer" effect (an increase in male GtH levels) and a "releaser" effect (a stimulation of male sexual behavior). Instead, we believe (Sorensen *et al.*, 1988a, b) that female goldfish release two distinct pheromones during the periovulatory period: a preovulatory (17,20β-P) pheromone with primer effects on the male endocrine system and a postovulatory (prostaglandin) pheromone which stimulates male reproductive behaviors but has no *direct* actions on male GtH levels and milt volume.

Although the increase in preovulatory 17,20β-P release by females coincides with increased blood GtH and milt volumes in males, the male responses appear earlier in the preovulatory period than our previous studies would have predicted. Dulka *et al.* (1987) found that when males were exposed to water-borne 17,20β-P, GtH levels increased within 15 min, although the milt volume was not increased for at least 3 hr. In the present study, however, both the GtH levels and milt volumes of the noncontact–ovulatory males first increased 12 hr prior to ovulation, at a time when 17,20β-P release from ovulatory females was apparently no different from that from nonovulators. The data from males sampled 9 hr prior to ovulation are also problematical because although 17,20β-P release from ovulatory females is by this time different from that from nonovulators, both GtH and milt of the two ovulatory male groups are significantly elevated, whereas the findings of Dulka *et al.* (1987) indicate the milt increase should not have occurred for at least another 3 hr. Finally, it is intriguing that GtH levels of ovulatory males rose gradually during the preovulatory period because when 17,20β-P is added to aquaria artificially (Dulka *et al.*, 1987), GtH reaches peak levels within 15 min.

One explanation for this apparently "early" milt response is that males might have been exposed to stimulatory 17,20β-P concentrations prior to the ovulatory GtH surge. Even 15 hr prior to ovulation, 6 hr before the onset of the 17,20β-P surge is evident, blood 17,20β-P levels of ovulatory females are distinctly higher than those of nonovulatory females. If 17,20β-P is released in urine, as is suggested for a steroidal sex pheromone in *Gobius jozo* (Colombo *et al.*, 1982), and if nudging of the ovipore by the courting male expresses pulses of urine, then ovulatory males may have been exposed to 17,20β-P levels sufficient enough to increase milt production over that of the nonovulatory males. Unlike the relatively chronic GtH elevation induced by the addition of 17,20β-P to aquarium water (Dulka *et al.*, 1987), 17,20β-P pulses encountered during courtship prior to the 17,20β-P surge may be transient, leading to the impression that milt volume increases without prior increases in GtH. An alternative explanation for the early milt increase is that preovulatory pheromonal activity is not restricted to 17,20β-P but is also exerted by related steroids such as 17P, which is capable of stimulating GtH and milt increase (Stacey and Sorensen,

1986 and unpublished), is known to be released in large quantities prior to ovulation (Van Der Kraak *et al.*, 1989), and could increase in the water prior to 17,20β-P. Further studies are required to explore these possibilities.

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